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(54) Title: SENSITIVE NUCLEIC ACID SANDWICH HYBRIDIZATION ASSAYS AND KITS

(57) Abstract

Nucleic acid sandwich hybridization assays are provided that incorporate one or a combination of background reduction steps. Those steps include use of a separate capture probe and separation from immobilized capture probes by cleavage and isolation. A very sensitive assay for RNA targets includes both of those steps, plus RNA binary probes, and RNA-directed RNA ligase and amplification by an RNA-directed RNA polymerase. Kits of reagents for performing assays according to this invention are also provided.

SENSITIVE NUCLEIC ACID
SANDWICH HYBRIDIZATION ASSAYS AND KITS

This invention relates to nucleic acid hybridization assays, more particularly sandwich hybridization assays using oligonucleotide hybridization probes. Such assays have broad applicability, including assays for mutant genes, assays for pathogens in biological samples, and assays for an organism or virus in food, agricultural products or the environment.

Background of the Invention

Oligonucleotide probes form extremely specific and stable complexes, known as "hybrids", with nucleic acid targets that contain target nucleic acid sequences complementary to the probes. Assays utilizing this principle generally have in common the steps of contacting nucleic acids in a sample with oligonucleotide probes under conditions that promote the formation of specific probe-target hybrids, washing away unhybridized probes, and measuring the number of probes that are hybridized (generally by measuring a signal generator associated with the probes) as an indication of the number of target nucleic acid sequences present (Gillespie & Spiegelman, 1965). For detection, a radioactive atom or a fluorescent moiety may be used. For detecting a small number of target molecules, the probes may contain an amplifiable group, such as an amplifiable reporter RNA (Chu et al., 1986), or

alternatively an amplifiable recombinant RNA that contains an
embedded probe sequence (Lizardi et al., 1988). If
bacteriophage Qβ replicase is used for amplification, an RNA
probe is amplified directly but a DNA probe may be amplified

5   indirectly, that is, the probe is transcribed to prepare an
RNA molecule that is then amplified directly (Martinelli et
al., 1992). In this application we refer to probes as being
"amplifiable" whether they are directly amplifiable or
indirectly amplifiable.

10   Sandwich hybridization assays are known (Dunn & Hassell,
1977; Ranki et al., 1983; Syvanen et al., 1986). In nucleic
acid sandwich assays, "capture probe" is used to bind the
target, to which a "reporter probe" may or may not is also
hybridized, to a solid surface. We sometimes refer to

15   reporter probes as "signal probes." The capture probe
hybridizes to the target at a site different from the "target
sequence" where the reporter probes hybridizes. The resulting
complexes include a target nucleic acid and a capture probe
also hybridized to the target and, possibly, reporter probe(s)

20   also hybridized to the target. The capture probe has one end,
the "head," hybridizable to the target and a second end, the
"tail," comprising a moiety that functions to bind the capture
probe to a solid surface, such as the surface of a particle.
Hybridization of the two probes is achieved by incubation with

25   the target in solution, using an excess, generally a large
excess, of both probes, followed by insolubilization, i.e.,
immobilization on a solid surface. Vigorous washing is used
to remove excess reporter probes not hybridized to targets
before detection of reporter probes.

Nucleic acid hybridization assays, including the sandwich
assays described above, suffer from poor sensitivity caused by
background or "noise" due to non-specific binding of reporter
probes to the particles or other solid surfaces. Pretreatment

5   of particles with albumin to block non-specific binding has
been used to reduce this background or noise, as has covalent
crosslinking of reporter probes to targets to permit more
stringent washing (Yabusaki et al., 1986). Also, probe-target
hybrids have been transferred from one solid surface to

10   another by a procedure known as "reversible target capture"
(Morrissey et al., 1989; Hunsaker et al., 1989). In
reversible target capture, the capture probe-target-reporter
probe complexes or hybrids are repeatedly released from
particles, transferred to a new container containing new

15   particles, recaptured (immobilized on the surfaces of the new
particles via the tails of the capture probes) and vigorously
washed. After a number of cycles of release, transfer,
recapture and washing, the number of reporter probes bound
nonspecifically to the final particles can be significantly

20   reduced. The overall process is so effective at removing
nonspecifically bound reporter probes that if a conventional
reporter probe containing a radioactive atom or fluorescent
moiety is utilized, no background can be detected (Thompson et
al., 1989). Reversible target capture is, however, complex

25   and time-consuming, machine-intensive and costly.

If amplifiable reporter probes (Lizardi et al., 1988) are
used in conjunction with reversible target capture, target
nucleic acids can be detected in samples at levels that are
lower than the levels that can be detected with conventional

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reporter probes (Lomeli et al., 1989). The resulting assays have been reported to be the most sensitive hybridization assays known (Pritchard & Stefano, 1991).

None of the assays that include the background-reduction approaches described above completely eliminates background. Even the most sensitive of those assays utilizing reversible target capture are not sufficiently sensitive to take full advantage of the power offered by amplifiable reporter probes. It has been shown, for example, that approximately 10,000 nonspecifically bound reporter probes persist, even after three cycles of reversible target capture (Lomeli et al., 1989). This limits the sensitivity of the assay to samples that contain 100,000 target molecules or more.

"Smart probes," that is, probes whose ability to generate a reporter signal depends upon hybridization properly to their targets (i.e., "target-dependent") have been proposed as a way to improve sensitivity. An example of a smart probe is one that contains a "molecular switch," wherein signal generation is dependent on a conformational change that takes place in the probe (Lizardi et al., 1992).

Another example of smart probes that have been disclosed is DNA "binary probes." A pair of DNA reporter probes hybridize adjacent to one another on the DNA target molecule, and one of the probes also functions to immobilize the hybrids on a solid surface, which permits washing. The probes can be ligated in a target-dependent fashion, namely ligation by a DNA-directed DNA ligase. The ligated product, a DNA "reporter molecule," may itself be detected, as when one reporter probe is used to immobilize the probe-target hybrids to a solid

surface and the other reporter probe contains a radioactive
atom or a fluorescent moiety (Landegren et al., 1988;
Landegren and Hood, 1991). Alternatively, the DNA reporter
molecule, immobilized on a surface as above by one of the

5   binary probes, may be amplifiable, either directly or
indirectly. Qβ replicase may be used to amplify the DNA
reporter molecule directly, or the DNA reporter molecule may
be transcribed, as by bacteriophage T7 RNA polymerase, to
produce an RNA transcript that is a template for an RNA-

10   directed RNA polymerase, such as Qβ replicase. In either
case, exponential amplification produces a detectable product,
whose detection is reported to indicate the presence of target
nucleic acid (Martinelli et al., 1992). In known binary-
probe assays, unhybridized immobilizing probes (i.e., one of

15   the DNA binary probes) will be bound to the solid surface,
just as unhybridized capture probes are in sandwich
hybridization assays that utilize unary reporter probes.

     Other assays use target-dependent, signal-generating
reactions. Such reactions include the ligase chain reaction,

20   LCR (Barany, 1991) the polymerase chain reaction, PCR (Erlich
et al., 1991), the multienzyme amplification reaction, 3SR
(Guatelli et al., 1990), the strand displacement amplification
reaction, SDA (Walker et al., 1992), and the target-dependent
replication reaction, TDR (Kramer & Lizardi, 1989).

25   It is an object of this invention to improve the
sensitivity of nucleic acid hybridization assays, preferably
to provide assays able to detect as few as 100 molecules of
target nucleic acid and, preferably, without background, so
that negative samples produce no signal.

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It is another object of this invention to provide highly sensitive nucleic acid hybridization assays that do not suffer from the drawbacks of reversible target capture, that is, complexity and cost, along with being time-consuming and

5   machine-intensive.

It is another object of this invention to provide highly sensitive assays compatible with exponential amplification to permit detection of rare target nucleic acids.

It is a particular object of this invention to provide

10 highly sensitive nucleic acid hybridization assays that can be performed without a sophisticated laboratory, even under field conditions. Assays that do not require particles but include a simple-to-use solid, such as a dipstick, are especially desired in this regard.

15   It is another object of this invention to provide nucleic acid hybridization assays of improved sensitivity that are quantitative as well as qualitative.

These and other objects of this invention will be apparent from the description that follows.

20

Summary of the Invention

In this description and in the appended claims, we sometimes refer to a molecule in the singular (e.g., "a capture probe"). The same applies to a complex (e.g., "a

25 probe-target hybrid"). It will be understood that many copies are in fact used or made. We sometimes utilize the plural rather than stating "copies of," which is cumbersome. Persons skilled in the art will understand the meaning from the context.

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The assays of this invention are sandwich nucleic acid
hybridization assays, by which is meant nucleic acid
hybridization assays that include immobilizing a target on the
surface of a solid and washing the solid. The sandwich

5   nucleic acid hybridization assays of this invention include at
least one improvement, but preferably a combination of
improvements to reduce background and improve sensitivity.
One necessary feature of the assays is the use of a capture
probe, by which we mean a probe that is not a reporter probe

10   and that is used to immobilize either targets or reporter
probe-target hybrids on the surface of a solid. More then one
capture probe may be used (by which is meant at least two
capture probes hybridizable to different sequences of the
target), but at least one must be used. As applied to the

15   instant invention, a "reporter probe" is limited to a probe
that does not function to immobilize reporter-probe targets on
a solid surface. Binary probe assays of the prior art do not
include the use of a capture probe. Rather, they use one of
the binary reporter probes as the means to immobilize reporter

20   probe-target hybrids on a solid surface. Thus, the binary
reporter probes used in the prior art binary probe assays are
not "reporter probes" as that term applies to this invention,
including the appended claims. We believe that this provides
an unappreciated source of significant background, because

25   many (typically 10^{10} to 10^{13}) copies of the immobilizing
reporter probe are not washed away, providing a high
concentration favoring target-independent ligation. For that
or some other reason, when a separate capture probe is used in
a binary-probe assay, a significant improvement results.

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A second feature of assays according to this invention, essential if targets alone or target-unary reporter probe hybrids are immobilized by capture probes and preferred if target-binary reporter probes hyrids are immobilized by capture probes, is the separation of targets or reporter probe(s)-target hybrids, as the case may be, from capture probes. Separation is accomplished by cleaving and then isolating the parts, that is, the target or reporter probe(s)-target hybrids, on the one hand. By "cleaving" is meant the breaking of on at least one phosphodiester bond at an appropriate site or sites in the target or in the capture probe.

We believe that retained capture probes are an unappreciated source of significant background, the many (typically 10^{13}) copies of the capture probe provide sites for non-specific binding of reporter probes. The presence of capture probes also may cause significant background if present during detection of the reporter molecule, by fluorescence. If binary probes are used, capture-probe separation is preferred but is not essential for all embodiments of this invention. Use of binary probes, a separate capture probe, and stringent washing or reversible target capture will satisfy the sensitivity requirements in some cases.

The sensitivity-enhancing improvements of this invention are widely applicable to nucleic acid sandwich hybridization assays. The target may be DNA or RNA. The reporter probe or probes may be DNA or RNA. And the capture probe may be DNA or RNA. Additionally, the capture probe may contain chemically

synthesized nucleotide analogues, such as 2'-O-methyl
nucleotides.

In embodiments including separation of reporter probe(s)-
target hybrids from capture probes, neither the nature of the

5   reporter probe or probes nor the method of generating a
detectable signal is critical. The probe may be unary or the
probes may be binary. A unary smart probe may be used. The
probe, or one of the binary probes, may contain a detectable
moiety such as a radioactive atom or fluorescent moiety, or

10   may include an enzyme that can be incubated with a colorless
substrate to generate large numbers of colored products (Leary
et al., 1983). Signal-generating techniques that utilize
exponential amplication are preferred. The reporter probe or
reporter molecule may be a template for an RNA-directed RNA

15   polymerase (Lomeli et al., 1989). Other exponential
amplication techniques that may be use are LCR, PCR, 3SR, SDA
and TDR.

In emobidments that include the separation of targets
from capture probes, which we fer to as "target purification,"

20   the assays must include a target-dependent signal-generating
technique from the group of LCR, PCR, 3SR, SDA and TDR.

The method of signal detection is not critical. Any
appropriate method of signal detection may be used, such as
radioactive counting or imaging, colorimetry, fluorescence or

25   luminescence. Signal detection may be "on the fly" or over
time for quantitative detection.

The type of solid surface on which the capture probe is
immobilized is not critical. The solid may be a particle, a
dipstick or the surface of a tube, for example. Any

appropriate immobilization technique may be used, depending on whether or not immobilization is to be permanent or reversible, as in reversible target capture.

In embodiments employing binary probes, ligating in a target-directed manner is required but the method is not critical. A protein or ribozyme ligase may be used, as well as a non-enzymatic method such as chemical ligation, photo-induced ligation or self ligation.

In binary-probe embodiments employing separation of capture probes, ligation may precede or follow separation, although we prefer that ligation follow separation. Further, the manner in which capture probes are isolated is not critical. Procedures for isolating a solid phase from a liquid phase are well known, including typically aspiration and decanting. Separation from the capture probe (or capture probes) preferably is used without reversible target capture, but a combination of techniques may be employed. Without reversible target capture, the capture probe or probes may be permanently linked to a solid surface, such as when the tail of the capture probe has a biotin moiety and when the surface of the particle has covalently linked streptavidin molecules or avidin molecules to bind the biotin.

In embodiments including separation from capture probes, preferred embodiments utilize cleavage with ribonuclease H ("RNase H"), which cleaves RNA that is hybridized to DNA. A DNA capture probe is utilized for an RNA target, in which case the target is cleaved. An RNA capture probe is utilized for a DNA target, in which case the capture probe is cleaved.

Preferred embodiments of the invention are assays for RNA targets, because RNA targets suitable for detection are in most cases much more abundant in samples than corresponding DNA targets.

5   For highly sensitive assays, exponential amplification of reporter probes, either directly or indirectly (after transcription) is preferred. Assays that utilize amplifiable reporter probes can theoretically detect as little as one target molecule, since a single amplifiable reporter probe can

10   serve as a template for the generation of billions of copies of itself (Levisohn and Spiegelman, 1968), leading to detection of the original reporter probe. The practical limit of detection is determined by the efficiency of the procedure that removes reporter probes that are not hybridized to target

15   molecules. The sensitivity of assays that utilize reversible target capture is limited by the persistence of nonspecifically bound reporter probes, as indicated above. By the present invention, the level of persistence of nonhybridized reporter probes is markedly reduced, permitting

20   assays of 1000-fold greater sensitivity and more.

Preferred embodiments of the invention utilize amplifiable binary probes; that is, probes that, when they are ligated to each other (and only when they are ligated to each other), form a reporter molecule that can be amplified

25   exponentially, or that serves as a template for the generation of a molecule that can be amplified exponentially. In preferred embodiments of the invention, the set of reporter molecules that can be formed by the ligation of two binary probes includes: 1) recombinant RNAs that can be directly

amplified exponentially by incubation with a compatible
RNA-directed RNA polymerase (Miele et al., 1983); 2) DNA
templates that comprise a promoter (or a sequence that can be
extended by incubation with a DNA polymerase to form a

5   promoter) and a sequence that encodes an RNA that can be
transcribed by a DNA-directed RNA polymerase that is
compatible with the promoter, so that the transcripts can then
be amplified exponentially by incubation with a compatible
RNA-directed RNA polymerase; and 3) DNAs or RNAs that can

10   serve as templates for an exponential amplification reaction,
such as LCR, PCR, 3SR, SDA and TDR.

       Preferred embodiments of the invention utilize solid
surfaces having streptavidin covalently linked to their
surface and utilize capture probes that contain a biotin

15   moiety so that the capture probes can be permanently bound to
the surface of the particles. While preferred embodiments
performed in our laboratory have utilized paramagnetic
particles as the solid, an aspect of cleavage-separation
according to this invention is that it permits assays that do

20   not require a well-equipped laboratory. Dipsticks or reaction
tubes (test tubes) may serve as the solid for an assay
particularly adapted for performance under field conditions.

       Preferred embodiments of the invention utilize binary
probes, a separate capture probe, and separation of the binary

25   probe-target hybrids from the capture probes (thus markedly
reducing target-independent hybrids that form from binary
probes that become nonspecifically bound to capture probes).
In these preferred embodiments, a single wash cycle is

sufficient to remove unhybridized binary probes, unhybridized cellular nucleic

acids, and all other cellular material. Preferably, separation precedes ligation.

5   Preferred embodiments of the invention are assays for RNA targets using binary probes made of RNA, and a target-directed RNA ligase as disclosed in U.S. application Ser. No. 08/004,993 for Diagnostic Assays and Kits for RNA Using RNA-Directed RNA Ligase, naming S. Tyagi as inventor (hereinafter Tyagi Ser.

10   No. 08/004,993) and U.S. application Ser. No 08/005,893 for Diagnostic Assays and Kits for RNA Using RNA Binary Probes and a Ribozyme Ligase, naming P. Lizardi, S. Tyagi, U. Landegren, F. Kramer and J. Szostak as inventors (hereinafter Lizardi et al. Ser. No. 08/005,893), both of which are being filed on the

15   same date as the instant application and both of which are incorporated herein by reference. Thus, one may use either a DNA-directed DNA ligase or a ribozyme ligase as the RNA-directed RNA ligase. Preferred as the DNA-directed DNA ligase is either bacteriophage T4 DNA ligase or E. coli DNA ligase.

20   Preferred as the ribozyme ligase is Tetrahymena ribozyme ligase (Doudna & Szostak, 1989). Another embodiment of the invention utilizes RNA targets, binary probes made of DNA, and a suitable DNA ligase, such as T4 DNA ligase.

        In preferred embodiments that utilize binary probes that,

25   when they are ligated to each other (and only when they are ligated to each other), form a reporter molecule that can be amplified exponentially, either directly or indirectly, it is preferable that the molecule that can be exponentially amplified be a template for an RNA-directed RNA polymerase.

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In preferred embodiments, the RNA-directed RNA polymerase is a bacteriophage RNA-directed RNA polymerase, such as Qβ replicase, MS2 replicase, or SP replicase. In the most preferred embodiment, the RNA-directed RNA polymerase is Qβ

5   replicase. In other preferred embodiments, the RNA-directed RNA polymerase is isolated from eucaryotic cells infected with a virus, such as cells infected with brome mosaic virus, cowpea mosaic virus, cucumber mosaic virus, or polio virus. In another preferred embodiment, the RNA-directed RNA

10 polymerase is T7 RNA polymerase (Konarska and Sharp, 1989).

In embodiments in which the molecule that can be exponentially amplified is a recombinant RNA that serves as a template for Qβ replicase, it is preferred that the sequence of the recombinant template be derived from the sequence of an

15 RNA in the following group: MDV-1 RNA, microvariant RNA, nanovariant RNA, CT RNA, RQ135 RNA, RQ120 RNA, and Qβ RNA. In the most preferred embodiment, the RNA is MDV-1 RNA.

In embodiments in which the molecule that can be exponentially amplified is a recombinant RNA that comprises an

20 MDV-1 RNA sequence into which a probe sequence has been embedded (Lizardi et al., 1988; and Lomeli et al., 1989), the probe sequence can be inserted at any location within the sequence of MDV-1 RNA. Although preferred locations for insertion of a sequence into MDV-1 RNA (and indeed for

25 insertion of a probe sequence into any other RNA template for an RNA-directed RNA polymerase) are those that occur in hairpin loops located on the exterior of the molecule in regions located away from regions that are required for replication (Miele et al., 1983), the probe sequence can be

inserted into any location and the resulting recombinant can be tested to see if it is exponentially replicatable. Methods for identifying potential hairpin loops that can serve as suitable insertion sites, methods for constructing the desired recombinant RNAs, and methods for testing the resulting recombinants to see whether they can be replicated exponentially by incubation with a compatible RNA-directed RNA polymerase, are known to those of ordinary skill in the art.

In preferred embodiments, the exponentially amplifiable recombinant RNAs contain an inserted probe sequence that is between 30 and 80 nucleotides long. In a most preferred embodiment, the exponentially amplifiable recombinant RNAs contain an inserted probe sequence that is about 40 nucleotides long. In preferred embodiments, the way in which the sequence of the exponentially amplifiable recombinant RNA is divided into "halves" for inclusion into the sequence of each binary probe, is that the sequence of the exponentially amplifiable recombinant RNA is divided about in the middle of the probe sequence. To illustrate this concept, imagine that the exponentially amplifiable recombinant RNA is based on a sequence that is 220 nucleotides long and the recombinant contains an embedded probe sequence that is 40 nucleotides long and that probe sequence is inserted between nucleotides 60 and 61 of the 220-nucleotide-long sequence (resulting in a sequence that is 260 nucleotides long). Then, in a preferred embodiment, the left probe would comprise a sequence that consists of the first 60 nucleotides of the 220-nucleotide-long sequence and immediately adjacent to it (at one end of the binary probe, for instance, the 3' end) would be the first

20 nucleotides of the 40-nucleotide-long embedded probe

sequence; the right binary probe would comprise the second 20

nucleotides of the 40-nucleotide-long embedded probe sequence

(at the 5' end of the binary probe) and immediately adjacent

5   to it would be the remaining 160 nucleotides of the

220-nucleotide sequence.

When the binary probes are made of RNA, and they are to

be synthesized by transcription from DNA templates, it is

preferable that the binary probe that has a probe sequence at

10   its 5' end begin with a guanosine, because guanosine is

preferred as the 5' nucleotide in syntheses that utilize known

DNA-directed RNA polymerases.

When the binary probes are made of RNA, and a ribozyme

ligase is utilized to link the binary probes, it is necessary

15   that the binary probe that has a probe sequence at its 5' end

contain an additional guanosine at its 5' end. This guanosine

is removed by the ribozyme when the binary probes are ligated

to each other.

When the binary probes are made of DNA and one of them

20   possesses a promoter (or a sequence that can be extended by

incubation with a DNA polymerase to form a promoter), and when

they are ligated together, the resulting DNA possesses a

sequence that encodes an RNA that can be transcribed by an RNA

polymerase compatible with the promoter, then there are a

25   number of preferred embodiments of how the binary probes

should be designed. These preferred embodiments include: 1)

a binary probe that encodes a promoter sequence should have a

hairpin structure at its 3' end, the stem of which either

comprises a (double-stranded) promoter or can be extended by

incubation with a DNA polymerase to form a (double-stranded) promoter, thus eliminating the requirement that a separate complementary DNA strand be present to hybridize to the sequence encoding the promoter to form a functional promoter;

5   2) it is preferable that when a binary probe contains a hairpin structure at its 3' end that comprises a (double-stranded) promoter, that the 3' end be used as a primer for its own extension by incubation with a DNA polymerase, resulting in the generation of a double-stranded

10   template for transcription by a compatible DNA-directed RNA polymerase, thus eliminating the inhibitory effects of the presence of hairpin structures on transcription (had the template remained single-stranded); and 3) it is preferred that a binary probe that encodes a promoter sequence should

15   not encode the region of the amplifiable recombinant RNA that comprises the binding site of the compatible RNA-directed RNA polymerase, resulting in a design that ensures that the short transcripts that can be synthesized from this binary probe (if it is not ligated) will not be able to be replicated.

20   And finally, in preferred embodiments of the invention, preferred methods of detecting include: 1) the incorporation of a radioactive nucleotide into RNA during amplification; and 2) the binding of a molecule, such as ethidium bromide or propidium iodide, that becomes fluorescent when it binds to

25   amplified RNA.

        Using amplifiable RNA binary probes, separate DNA capture probes, cleavage of the reporter probes-target hybrids from the capture probes by incubation with RNase H and isolation of the reporter probes-target hybrids from particles containing

the capture probes, an assay according to this invention has

been demonstrated to detect, qualitatively and quantitatively,

RNA target molecules in amounts ranging from 100 to 10,000,000

molecules per sample, and as few as one HIV-1-infected human

5   lymphocyte cell in a sample containing 100,000 uninfected

human lymphocyte cells with no background signal for samples

containing no target molecules and for samples containing no

infected cells. This level of sensitivity is achieved without

resort to reversible target capture.

10   This invention also includes diagnostic kits for

preselected targets. Kits according to this invention may

contain a unary reporter probe, at least one capture probe, a

cleaving agent, and instructions for carrying out a diagnostic

assay. Kits may include a pair of binary probes, at least one

15   capture probe and instructions. Preferred kits include a pair

of binary probes, at least one capture probe, a cleaving agent

and instructions. Preferred kits may also include enzymes

(e.g., RNase H, T4 DNA ligase and Qβ replicase) and,·

optionally, other reagents.

20   A detailed description of numerous embodiments of this

invention, including particularly preferred embodiments,

follows in the Detailed Description. Neither the Detailed

Description nor the examples are intended to limit the scope

of the invention or the claims to the preferred embodiments.

25

Brief Description of the Drawings

Fig. 1 depicts the assay of Example 1.

Fig. 2 shows the sequences of the probes of Example 1.

Fig. 3 depicts the preparation of binary probes of Example 1.

Fig. 4 shows typical results of an assay according to Example 1 for known amounts of target molecule.

5   Fig. 5 shows the results of an assay according to Example 1 for known amounts of infected cells.

Fig. 6 shows the results of another assay according to Example 1 for known amounts of infected cells.

Fig. 7 depicts the assay of Example 2.

10   Fig. 8 shows the sequences of the probes of Example 2.

Fig. 9 depicts the assay of Example 3.

Fig. 10 depicts the assay of Example 4.

Detailed Description

15   As stated, this invention is broadly applicable to nucleic acid sandwich hybridization assays utilizing reporter probes and separate capture probes, that is, capture probes that are not reporter probes.

At least one capture probe is used, optionally more than 20   one, as in our most preferred, highly sensitive assay described below in Example 1. The use of two capture probes rather than one increases the chance that a given target molecule will be captured, because the act of capturing is not 100% efficient.

25   It is desirable that the capture probe-target hybrids be stable, so that hybrids are not lost during the assay. Persons skilled in the art are able to select a hybrid length sufficiently long to withstand the processing conditions of a particular assay. Generally, we prefer capture probe-target

hybrid lengths of 30-50 basepairs, which defines the length of

the head (in nucleotides) of the capture probe. The tail of

the capture probe or probes is used to immobilize the capture

probe and its hybrids to a solid surface, either permanently

5   or reversibly. If separation of the capture probe or probes

according to this invention is used, we prefer that the tail

of the capture probe be a biotin group that will react with a

streptavidin-coated solid surface. Between the head of a

capture probe and its tail is a spacer, generally an

10 oligonucleotide sequence. We prefer assays in which

hybridizations and ligations are performed in solution, that

is, when targets are not immobilized on a solid surface.

Particularly for such preferred assays, the spacer may be

quite short. Preferred spacers are 3-8 nucleotides in length.

15   The cleavage step and the cleaving agent or agents that

are appropriate depend on the nature of the target nucleic

acid, the nature of the capture probe, and the nature of the

reporter probes, if present. In general, there are eight

different situations that can occur. Table 1 below identifies

20 each situation by a Roman numeral:

Table 1

Situation	Target	Capture Probe	Reporter Probe
I	RNA	RNA	RNA
II	RNA	RNA	DNA
III	RNA	DNA	RNA
IV	RNA	DNA	DNA
V	DNA	RNA	RNA
VI	DNA	RNA	DNA
VII	DNA	DNA	RNA
VIII	DNA	DNA	DNA

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The following nine cleavage techniques illustrate the
variety of ways that reporter probe-target hybrids can be
released from capture probes by cleaving agents, with
reference to the situations in Table 1.

5   1. Cleavage of an RNA target with a single-strand-
specific ribonuclease (such as ribonuclease A or ribonuclease
T1) (situation IV). Because these ribonucleases do not cleave
DNA, nonspecifically bound reporter probes are not released
from the capture probes. An embodiment of this technique is

10   described in Example 6. This cleavage technique is not
applicable to target purification.

        2. Site-specific cleavage of an RNA target with a
ribozyme (situations I, II, III and IV). Because the ribozyme
can be designed to cleave a particular site in the target RNA

15   (Haseloff & Gerlach, 1988), a third element of specificity is
added to the assay. Cleavage of the target RNA is designed to
occur in the region between the sites in the target where the
capture probe and the reporter probe bind, thus releasing the
reporter probe-target hybrid from the solid surface. Two

20   embodiments of this technique are described in Example 3.

        3. Site-specific cleavage of an RNA target with
ribonuclease III ("RNase III"), utilizing a site-identifying
probe made of RNA (situation IV). The site-identifying probe
is designed to be complementary to a pre-selected site in the

25   target that occurs in the region between the sites where the
capture probe and the reporter probe bind. The site-
identifying probe is included in the original hybridization
mixture. Because ribonuclease III only cleaves double-
stranded RNA at particular sequences (Krinke & Wulff, 1990), a

third element of specificity is added to the assay. Cleavage

of the target RNA occurs in the region between the sites where

the capture probe and the reporter probe bind, thus releasing

the reporter probe-target hybrid from the solid surface. We

5　found experimentally that ribonuclease III will cleave almost

all double-stranded RNAs if they are sufficiently long. For

this reason, the use of ribonuclease III in situations I, II,

III is not preferred. An embodiment of this technique is

described in Example 4.

10　　　　4. Site-specific cleavage of an RNA target with

ribonuclease H, utilizing a site-identifying probe made of DNA

(situation I). The site-identifying probe is designed to be

complementary to a pre-selected site in the target that occurs

in the region between the sites where the capture probe and

15　the reporter probe bind. The site-identifying probe is

included in the original hybridization mixture. Because

ribonuclease H only cleaves RNA that is hybridized to DNA,

cleavage is limited to the site where the site-identifying

probe binds. The use of a site-identifying probe introduces a

20　third element of specificity into the assay. Cleavage of the

target RNA occurs in the region between the sites where the

capture probe and the reporter probe bind, thus releasing the

reporter probe-target hybrid from the solid surface.

　　　　5. Site-specific cleavage of a DNA target with a

25　restriction endonuclease, utilizing a site-identifying probe

made of DNA (situations V, VI, VII and VIII). The site-

identifying probe is designed to be complementary to a pre-

selected site in the target that occurs in the region between

the sites where the capture probe and the reporter probe bind.

The site-identifying probe is included in the original
hybridization mixture. Because restriction endonucleases only
cleave double-stranded DNA at particular sequences, a third
element of specificity is added to the assay. Cleavage of the

5   target DNA occurs in the region between the sites where the
capture probe and the reporter probe bind, thus releasing the
reporter probe-target hybrid from the solid surface.

6. Cleavage of an RNA target with ribonuclease H at the
site where the head of a DNA capture probe is bound (situation

10   III). Because ribonuclease H only cleaves RNA that is
hybridized to DNA, cleavage is limited to this specific site.
By appropriate design of the capture probe, as persons skilled
in the art will readily appreciate, cleavage of the target RNA
destroys the capture probe-target hybrid, releasing the

15   reporter probe-target hybrid from the solid surface. An
embodiment of this preferred technique is described in Example
1.

7. Cleavage of an RNA capture probe with ribonuclease H
at the site where a DNA target is bound (situation VI).

20   Because ribonuclease H only cleaves RNA that is hybridized to
DNA, cleavage is limited to this specific site. By
appropriate design of the capture probe, as persons skilled in
the art will readily appreciate, cleavage of the head of the
capture probe destroys the capture probe-target hybrid,

25   releasing the reporter probe-target hybrid from the solid
surface. An embodiment of this technique is described in
Example 8.

8. Cleavage of an RNA target with ribonuclease III at
the site where the head of an RNA capture probe is bound

(situation II). Because ribonuclease III only cleaves double-
stranded RNA, cleavage is limited to this specific site.
Cleavage of both strands of RNA in the capture probe-target
hybrid releases the reporter probe-target hybrid from the
solid surface. An embodiment of this technique is described
in Example 5.

9. Cleavage of a DNA target with a restriction
endonuclease at the site where the head of a DNA capture probe
is bound (situations VII and VIII). Cleavage of both strands
of DNA in the capture probe-target hybrid releases the
reporter probe-target hybrid from the solid surface. An
embodiment of this technique is described in Example 7.

The assays of this invention utilize certain assay
techniques that will be readily understood by persons skilled
in nucleic acid sandwich hybridization assays.

The techniques will be described for embodiments in which
reporter probe(s)-target hybrids are separated from capture
probes. A large excess of reporter probes and capture probes
is added to a sample under conditions promoting hybridization.
Hybridization preferably occurs in solution. Pre-
immobilization of capture probes on a solid surface is not
preferred but is not excluded. Immobilization via the tails
of the capture probes follows, after which the supernatant is
removed and the solid washed, preferably vigorously, and a
clean supernatant is added. When used, cleavage releases
reporter probe-target hybrids into the supernatant, which is
then isolated from the solid containing the capture probes, as
by decanting or aspiration. When binary probes are used, they
are ligated, preferably at this point in the assay.

Amplification, if desired, and detection follow. Several

detection methods are well known, including radiation (use of

a radioactive nucleotide triphosphate), colorimetric (e.g.,

use of an enzyme that can cause a color change in a substrate)

5   fluorescence (e.g., use of a dye such as propidium iodide),

and luminescence (e.g., use of an alkaline phosphatase

substrate that releases photons upon cleavage). Detection can

be qualitative or quantitative, as will be discussed below.

Preferred embodiments utilize isolation procedures in

10   which the capture probes that are bound to the particles can

be physically removed from the solution containing the

released reporter probe-target hybrids. If non-paramagnetic

particles are used, a centrifuge can be used to force the

particles to the bottom of the test tube and the supernatant

15   containing the reporter probe-target hybrids can be removed by

aspiration; or if the particles are paramagnetic (which is

preferred because a centrifuge is not needed), a magnet can be

used to draw the particles to the walls of the test tube and

the supernatant containing the reporter probe-target hybrids

20   can be removed.

Preferred embodiments of the invention utilize

paramagnetic particles containing streptavidin covalently

linked to their surfaces and capture probes that contain a

biotin moiety at their tails (so that the capture probes can

25   be permanently linked to the surface of the particles or

beads).

Preferred embodiments of assays according to this

invention, particularly for highly sensitive assays, are

assays for RNA targets and utilize RNA reporter probes, which

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have the potential of generating an easily detectable
amplified signal from even a single reporter molecule, at
least one DNA capture probe and ribonuclease H.

Particularly preferred embodiments of the invention use
5   reporter probes that are binary hybridization probes, neither
of which functions to immobilize the target on a solid
surface, which can only be ligated to each other in a target-
dependent reaction if they are correctly hybridized adjacent
to one another, and which, when they are ligated, form a
10   recombinant reporter molecule that is exponentially
amplifiable by Qβ replicase, either directly (serving as a
template for the replicase) or indirectly (serving as a
transcription template, the transcripts then serving as
templates for the replicase). Most preferred are assays for
15   RNA targets utilizing DNA capture probes and RNA binary
probes. The methods of this invention are particularly
attractive when using binary hybridization probes, wherein the
ligation and amplification reactions are carried out without
interference from the presence of solid surfaces. In
20   contradistinction, previous methods that utilize binary
hybridization probes utilized one of the binary probes to
immobilize probe-target hybrids (Landegren et al., 1988; and
Martinelli et al., 1992); and these methods suffer from the
presence of solid surfaces during ligation.

25   One important objective of this invention is an assay
that will detect very few target molecules, as few as 100 in a
sample, without a background signal from negative samples.
Example 1 illustrates a preferred embodiment of such an assay.
The assay of Example 1 is an assay for an RNA target utilizing

RNA binary reporter probes whose ligation produces an RNA reporter molecule that is directly amplifiable by an RNA-directed RNA polymerase, in this case $Q\beta$ replicase. Use of a separate capture probe permits washing away unused copies of

5 both binary reporter probes before ligation of the binary reporter probes. Ligation is target-dependent. Target-independent ligation, which we believe to be a source of background, is greatly reduced by washing away both binary reporter probes rather than having one binary reporter probe

10 immobilized on a solid surface in high concentration.

Even so, nonspecifically bound reporter probes remain as a source of background. Reversible target capture is one technique that may be used to reduce this source of background to a low level that will be acceptable for many assays.

15 However, reversible target capture has significant drawbacks. First, it is costly, time-consuming, complex and machine intensive. Second, there is the possibility of losing some reporter probe-target hybrids in every round of reversible target capture. Third, we believe that capture probes

20 themselves provide sites for non-specific binding of reporter probes. In reversible target capture all capture probes, the few bound to targets and the great excess not bound to targets, are retained.

The assay of Example 1 addresses all of those drawbacks.

25 Reporter probe-target hybrids are separated from both capture probes by cleaving the reporter probe-target-capture probe hybrids with ribonuclease H. This cleavage releases the reporter probe-target hybrids and leaves the capture probes, and reporter probes nonspecifically bound thereto, behind on

the surface of the solid (in Example 1 the solid is paramagnetic particles), permitting an easy physical separation. Ligation is performed after this separation. Amplification directly with Qβ replicase and detection follow.

By this combination of steps, the background due to nonspecific hybridization was reduced to zero. The resulting assay was sufficiently sensitive to detect as little as 100 target nucleic acid molecules. And finally, and most significantly, by combining the use of a smart probe, e.g., binary reporter probes, and a separate capture probe with a step that separates reporter probe-target hybrids from capture probes, it was possible to carry out these extremely sensitive assays without resort to the costly, time-consuming, complex, and machine-intensive reversible target capture procedure. Consequently, sensitive hybridization assays can be carried out on dipsticks, utilizing simple washing techniques that are commonly used in clinical immunoassays.

As stated earlier, this invention also includes kits for performing assays according to this invention. A preferred kit may contain some or all of the following items:

1. 5 M guanidine thiocyanate (GuSCN) buffer for the lysis of cells in clinical samples;

2. A mixture containing capture probes and reporter probes, preferably binary probes, for a preselected nucleic acid target;

3. A solid, such as a dipstick, reaction tube or paramagnetic particles, with streptavidin covalently bound thereto;

4. A magnetic separation device;

5. Nucleotides;

6.   Ribonuclease H, T4 DNA ligase and Qβ replicase;

7.   Buffers for ligation and amplification;

8.   Reagents for detecting the amplified reporters, such as radioactive alpha-P^{22}-cytidine triphosphate or propidium iodide;

9.   If the binary probes are made of DNA, the Klenow fragment of E. coli DNA polymerase and T7 RNA polymerase; and

10.   Instructions for performing an assay according to this invention.

A bare kit may contain only binary reporter probes, at least one capture probe and instructions, items 2 and 10. Another kit may contain a unary reporter probe, at least one capture probe and a cleaving agent, preferably ribonuclease H, as well as instructions, items 2, 6 and 10. A more complete kit for preferred assays will contain binary reporter probes, at least one capture probe, plus at least items 1, 3, 6 and 10, as well as item 9 when appropriate. A kit containing at least items 1-3 and 5-10 (item 9 only where appropriate), where the solid is a dipstick or reaction tube, is particularly useful for assays to be performed outside a well-equipped laboratory.

As stated earlier, certain embodiments of this invention are types of nucleic acid sandwich hybridization assays that include target purification.

The description in the preceding paragraphs regarding capture probes, cleavage steps and isolation steps in equally applicable to target purification with the exception that reporter probes need not be taken into account, because they are not present during target purification. Preferred targets for assays including target purification according to this

invention are RNA targets, because RNA targets suitable for detection are in most cases much more abundant in samples than corresponding DNA targets.

When LCR follows target purification, we especially prefer the use of RNA probes and an RNA-directed ligase, such as T4 DNA ligase, (a protein molecule,) or _Tetrahymena_ ribozyme ligase (an RNA molecule.) If the latter is used, the LCR probes have hybridization lengths no longer than aout nine nucleotides.

A preferred kit for an assay according to this invention that includes target purification may contain some or all of the following items:

1.   5 M GuSCN;

2.   DNA capture probe(s) for a preselected RNA target;

3.   A solid, such as paramagnetic particles, coated with streptavidin;

4.   A magnetic separation device;

5.   Ribonuclease H;

6.   RNA LCR primers;

7.   T4 DNA ligase;

8.   LCR buffer; and

9.   Instructions.

A complete kit will include all of items 1-9. A bare kit will include at least items 2, 6 and 9. A universal kit for target purification in embodiments of assays according to this invention will include at least items 2 and 9, preferably also items 3 and 5.

EXAMPLES

Example 1: RNA Target, RNA Binary Probes, DNA Capture Probes and Ribonuclease H Cleavage

The present invention reduces the background in sandwich hybridization assays, while simultaneously simplifying the format of the assays. This embodiment, which employs RNA binary probes, has led to extremely sensitive assay depicted generally in Figure 1 for the detection of an RNA target, illustrated here by human immunodeficiency virus ("HIV") RNA. The target sequence 2 of target 1 is located in the integrase region of HIV RNA. The assay begins by dissolving the sample in 5 M guanidine thiocyanate (GuSCN). A mixture of four different nucleic acid probes is then added to the sample. These probes hybridize to the HIV RNA, as shown in Figure 1. Two of the probes are capture probes 3, 4 made of DNA. Each capture probe has a hybridization sequence 5 or 6 at its 3' end that is complementary to the target 1. Capture probes 3, 4 also have a biotin moiety 7 at their 5' ends. The other two probes 8, 9 are binary reporter probes made of RNA. After hybridization, the hybrids are captured on the surface of paramagnetic particles 10 coated with streptavidin 11, which binds tightly to the biotin moiety 7 of the capture probes. The paramagnetic particles 10 are then washed extensively to remove unhybridized binary probes. RNase H is then added to cleave the target RNA in the region where it is hybridized to the capture probes. Cleavage of the target RNA frees the binary probes-target hybrids 12 (shown in Figure 1 after ligation) from the capture probes.

The cleavage release step is quantitative and specific. The binary probes that nonspecifically adhere to the DNA capture probes or to the surface of the paramagnetic particles (i.e., sources of the background signal) are not released by

5   this cleavage. The paramagnetic particles 10 are discarded. Assays utilizing this target-dependent release achieve, in a single step, a signal-to-noise ratio that is similar or superior to the signal-to-noise ratio achieved in the multi-step reversible target capture method.

10   In the supernatant, pairs of binary probes that are correctly hybridized adjacent to one another on their targets are ligated to each other in a target-dependent manner. The ligated probes 13 are then amplified by incubation with Qβ replicase. The amplified signal is strictly dependent on the

15   presence of the target, and the level of the signal as a function of time can be used to determine quantitatively the number of molecules of HIV RNA in the initial sample. By combining a target-dependent RNase H-mediated release step with a target-dependent ligation step, background signal is

20   completely eliminated.

A.   Synthesis of the Probes

The capture probes used in this example (Figure 2) have three functional parts. A head of 40-50 nucleotides which hybridize to the preselected target, a spacer of about 4

25   nucleotides, and a tail that binds tightly to the solid surface. We chose the tail to be a biotin moiety and covalently linked it to the 5′ end of each capture probe. The biotin moiety can be attached anywhere in the capture probe, including at the 3′ end and internally. The tail can be made

up of some other affinity reagent, such as a
homopolynucleotide.

Figure 1 shows the manner in which capture probes 3, 4
bind to target 1. Two different capture probes were used,
5   rather than one, in order to increase the efficiency of
capture and increase the stringency of release of the binary
probe-target complexes. The two capture probes 3, 4 bind to
target 1 on either side of the target sequence 2 to which the
binary probes bind. Hybridization sequence 5 of capture probe
10   3 is complementary to region 4415-4458 of HIV genomic RNA, and
hybridization sequence 6 of capture probe 4 is complementary
to region 4808-4852 of HIV genomic RNA. Figure 2 shows the
sequences of the two capture probes 3, 4 used in this example.
Underlines indicate hybridization sequences 5, 6 that are
15   complementary to the target RNA. Both of the capture probes
were prepared on a DNA synthesizer.

The desired reporter molecule of this particular
embodiment requires the ligation of two binary probes, as
shown in Figure 1. The use of binary probes is a preferred
20   embodiment. Figure 1 illustrates the design of binary
reporter probes 8, 9. The 5' end 14 of probe 8 consists of
the first sixty-nine nucleotides of the replicatable probe for
HIV used in our previous studies (Lomeli et al., 1989). The
next twenty-three nucleotides are probe sequence 15, which is
25   complementary to region 4596-4618 of HIV genomic RNA. The 5'
end of probe 9 consists of 19-nucleotide probe sequence 17,
which is complementary to region 4577-4595 of HIV genomic RNA.
The remainder 16 of probe 9 corresponds to nucleotides 95-280
of the replicatable probe for HIV used in our previous studies

(Lomeli et al., 1989). Figure 2 shows the sequences of binary probes 8, 9. Underlines indicate hybridization sequences 15, 17 that are complementary to the target sequence. Neither of these molecules is a good replicator when incubated with Qβ

5   replicase, but if they are ligated to each other, they form an exponentially replicatable reporter molecule.

Binary probes 8, 9 were prepared by transcription from DNA templates generated in a polymerase chain reaction (PCR) shown in Figure 3. The plasmid 20 described in Lomeli et al.,

10   1989 at page 1827, which we call plasmid pT7MDVHIV20, was used as the source of MDV sequences in the PCR reactions. The relevant section of the plasmid is shown in Figure 3. It is, of course, double-stranded. Four PCR primers 21, 22, 23 and 24 were designed in such a way that they contributed

15   additional sequences to the PCR products 27, 28 that were not present in plasmid 20, but which were required. PCR product 27 is generated from primers 21, 22. PCR product 28 is generated from primers 23, 24. Primer 22 provided the terminal twenty-three nucleotides 25 in the template for probe

20   8. Primer 23 provided T7 promoter 26 at the 5' end of the template for probe 9. (Plasmid 20 provided a T7 promoter in the region of primer 21.) Probe 8 was transcribed in the usual manner from its PCR template, but the synthesis of probe 9 required a modification in the conditions of transcription

25   by T7 RNA polymerase. The donor of the phosphate group in a ligation reaction, in this case probe 9, must contain a single phosphate at its 5' end. RNA molecules prepared by transcription usually have a triphosphate at their 5' ends. In order to synthesize probe 9 with a single phosphate group

at its 5' end, a ten-fold excess of guanosine 5'-monophosphate
over the nucleoside 5'-triphosphates was included in the
transcription reaction. This lead to the incorporation of
guanosine 5'-monophosphate at the first position of the probe

5 9. As a result, the copies of probe 9 possessed the required
monophosphate at their 5' ends. After transcription, each of
the binary probe RNAs 8, 9 was purified by preparative
polyacrylamide gel electrophoresis. The RNA was eluted
directly from the gel slice into a 2 M guanidine thiocyanate

10 (GuSCN) solution.

We have discovered since this example was performed that
probe 9 can be improved by using a twenty-fold excess of
guanosine 5'-monophosphate rather than the ten-fold excess
reported above. Efficiency of ligation triples, from 10% to

15 30%, when the greater excess is used.

B. Hybridization, Capture, Washing, and Release

The assay of this example has been performed on samples
containing known amounts of HIV RNA molecules. An RNA
corresponding to the mRNA of the integrase gene of HIV was

20 used as a model target. This RNA was prepared by
transcription from linearized plasmid pGEM-integrase, that was
prepared in our laboratory. Eight tubes containing differing
amounts, for example, 10000000, 1000000, 100000, 10000, 1000,
100, 10, and 0 molecules, of integrase RNA in 50 microliters

25 of 2 M GuSCN were prepared by serial dilution. A 2 M GuSCN
solution (50 microliters) containing 10^{13} molecules of each of
the capture probes and 2×10^{10} molecules of each of the binary
probes was added to each tube. Hybridization was carried out
by incubation at 37 degrees centigrade for one hour. A

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30-microliter suspension of paramagnetic particles coated with
streptavidin (Promega) was then added to this hybridization
mixture. The probes-target hybrids were captured on the
surface of the paramagnetic particles by a 10-minute

5   incubation at 37 degrees centigrade. The particles were
washed with 2 M GuSCN four times, with 300 mM KCl three times,
and finally with 1X ligase buffer (66 mM Tris-HCl, pH 7.5, 5
mM MgCl$_2$, 1 mM DTT, 1 mM ATP) two times. After washing, one
unit of E. coli RNase H (Pharmacia) dissolved in 50

10   microliters of 1X ligase buffer was added. The binary
probes-target hybrids were released from the surface of the
paramagnetic particles by a 10-minute incubation at 37 degrees
centigrade. The tubes containing the mixture were placed in
the magnetic field provided by a magnetic separation device to

15   draw the paramagnetic particles to the walls of the test
tubes. The supernatant was then separated from the
paramagnetic particles by aspiration and placed into a fresh
tube. The probe-target hybrids were then incubated with T4
DNA ligase in order to ligate the binary probes that were

20   correctly hybridized to targets. Ligation was carried out in
40 microliters of the supernatant by the addition of 40 units
of T4 DNA ligase and incubation at 37 degrees centigrade for
one hour.

The assay of this example has also been performed on

25   samples containing known amounts of HIV-infected cells. In
order to demonstrate the specificity and sensitivity of this
assay with clinical samples, human peripheral lymphocytes were
infected with HIV. These infected cells were serially diluted
with uninfected human peripheral lymhocytes. Eight tubes were

prepared, containing differing amounts, for example, 600000, 60000, 6000, 600, 60, 6, 0, and 0 infected cells. Each of these tubes had the same total number of cells, for example, 600000. The tubes were centrifuged and the supernatant was

5   removed. 240 microliters of 5 M GuSCN was added to each tube. The tubes were then incubated for 2 hours at 37°C in order to lyse the cells. After lysis, 40 microliters from each tube were assayed. A 60 microliter solution containing all four probes was added to the lysate. The addition of this solution

10   reduced the GuSCN concentration in the lysate to 2 M. Hybridization and all subsequent reactions were carried out the same as described in the previous paragraph.

C.   Amplification

The reporter molecules, comprising ligated binary probes,

15   were then amplified by incubation with Qβ replicase. It is not necessary to melt apart the reporter molecule-target hybrids prior to amplification. A mixture containing all of the components of the replication reaction was added to each of the eight tubes. The final reaction mixture (120

20   microliters) was 45 mM Tris-HCl (pH 8), 10 mM MgCl$_2$, 400 micromolar ATP, 400 micromolar GTP, 400 micromolar UTP, 400 micromolar alpha-P^{32}-CTP, and contained 50 micrograms per milliliter Qβ replicase. Each reaction was incubated at 37 degrees centigrade, and 4-microliter samples were withdrawn

25   from each reaction every minute in the interval between 10 to 31 minutes of incubation. Each sample was mixed with a 45-microliter stop solution (120 mM NaCl, 20 mM EDTA, and 3 microgram per ml proteinase K). This solution stops replication by sequestering the required magnesium ions. The

stop solutions were arranged in titerplates before addition of the samples. The RNA in each stopped reaction was separated from the unincorporated nucleoside triphosphates by precipitating the RNA in an acidic solution (360 mM phosphoric acid, 20 mM sodium pyrophosphate, and 2 mM EDTA), trapping the precipitate on a blotting membrane (Zeta Probe, Biorad), and then washing the membrane with the acidic solution. The RNA on the blots was visualized by autoradiography.

D. Results

The results demonstrate that it is possible to achieve very high sensitivity with no background. Figure 4 shows a typical result of the assay described above for a dilution series of molecules of HIV integrase RNA. Figure 4 is an autoradiogram. Each row depicts the signal from a given sample over time, as indicated. The intensity of each dot in Figure 4 is proportional to the amount of RNA that was present in the tube at the time the sample was withdrawn. The time at which RNA can first be seen in the autoradiogram depended upon the number of targets that were present. The greater the number of targets, the earlier the signal appeared. There is a delay of at least about two-minutes for every 10-fold decrease in the number of targets. As few as a 100 targets molecules gave rise to a clear signal. Ten target molecules, or no target molecules, did not produce a signal, even after an additional eight minutes of incubation (not shown). One hundred molecules of HIV RNA target were clearly detectable; and anything less gave no signal at all.

As stated, the minimum number of targets that could be detected was a hundred molecules. However, the number of

ligated replicatable RNA molecules in the corresponding tube
prior to replication was considerably less. We have
experimentally determined that the number of probe-target
complexes remaining after hybridization, capture, washing, and
5   release is 30 percent of the original number of targets
present. Furthermore, we have determined that the efficiency
of ligation was 10 percent. Therefore, in the tube that
originally contained 100 target molecules, only 3 replicatable
RNA molecules were likely to be present. Since at least 1
10   replicatable RNA molecule is required to initiate an
amplification reaction, samples containing less than 100
molecules are unlikely to generate a signal. Since an
infected clinical sample is likely to contain at least 1,000
target molecules (corresponding to a single infected cell),
15   this extremely sensitive assay has adequate sensitivity for
virtually all clinical applications. The improvement in
synthesis of probe 9 reported above, which triples ligation
efficiency, will improve the sensitivity of this assay even
more.

20   Figures 5 and 6 show the results of two experiments
designed to demonstrate the clinical utility of this
invention. They depict the results of assays according to
this example on samples containing HIV-infected human
peripheral lymphocytes. Figures 5 and 6 are autoradiograms as
25   described in connection with Figure 4. The results of the two
experiments, when considered together, show that one infected
cell can be detected in a sample containing 100,000 uninfected
cells, and that no signal is seen in samples that contain no
infected cells. In both experiments each sample contained a

total of 100,000 cells. In the first experiment (Figure 5), the results show a clear relationship between the number of infected cells in a sample and the time of appearance of the signal. The greater the number of infected cells, the earlier

5   the signal appeared. Thus, the assay is quantitative as well as qualitative. The two controls, each of which contained 100,000 uninfected cells (and no infected cells), produced no signal. However, the tube that contained one infected cell also did not give a signal. We hypothesized that, after

10 lysis, the sample was not mixed completely. The lysed samples are extremely viscous (due to the presence of cellular DNA), and since there were only about six infected cells in the 240 microliter volume of the lysate, the probability of not sampling any HIV RNA in the 40 microliter sample withdrawn

15 from the sample for the assay was high. In order to eliminate such a possibility, the samples were frozen and thawed and then exhaustively mixed, prior to using them in the next experiment. The assay was repeated with these well-mixed samples. The results, shown as the bottom six samples in

20 Figure 6, demonstrate that our hypothesis was correct and that, after proper mixing, the tube containing the nucleic acid from about one infected cell gave a strong signal with an appropriate delay time.

         Another objective of the second experiment was to

25 estimate the number of molecules of HIV RNA in each infected cell. We prepared two standards for this purpose. Standard 1, the first standard tube, contained 1,000,000 HIV integrase mRNA transcripts. Standard 2, the second standard tube contained a lysate from 100,000 uninfected cells and 1,000,000

molecules of HIV integrase mRNA transcripts. The results of
the experiment, shown as the top two samples in Figure 6, show
that the presence of lysate from the uninfected cells has only
a slight quenching effect. From the signal obtained from this
internal standard, we were able to estimate that each infected
cell contained about 3,000 HIV target molecules.

A note of caution is in order. Our laboratory is used
for research on replicatable RNA molecules, which have a
tendency to become airborne contaminants. In a test of the
above assay, we obtained an aberrant signal in one of the
tubes. Electrophoretic analysis of the amplified RNA revealed
that it was not the reporter. Qβ replicase will amplify a
number of RNAs, and our sample had become contaminated.

Example 2: DNA Target, DNA Binary Probes, RNA Capture Probes
and RNase H Cleavage

This preferred embodiment, depicted generally in Figure
7, is for target DNA 51 and utilizes binary probes 58, 59 made
of DNA. In this example, the capture probes 53, 54 are made
of RNA, with a biotin moiety, in this case a terminal biotin
moiety 57, for immobilization on a solid surface. The
sequence of the capture probes is shown in Figure 8. Each
binary probe is a portion of a DNA template that can be
transcribed into an amplifiable RNA. Binary probe 58 contains
at its 5' end 64 twenty-two nucleotides that can hybridize to
HIV RNA 51 in the region 4532-4553, part of target sequence
52. Adjacent to this probe sequence 64 are sixty nucleotides
63 that encode the 5' end of MDV-1 (+) RNA. The 3' end of the
probe 58 forms a hairpin structure 60. When the 3' end of

this DNA is extended by incubation with the Klenow fragment of

E. coli DNA polymerase, the resulting double-stranded DNA

contains a promoter for T7 RNA polymerase directed towards the

MDV-1 sequence. Figure 8 shows the sequence of probe 58,

5   including hairpin structure 60. Box 67 shows what will be the

T7 promoter once the 3' end of probe 58 is extended to "fill

the box" 67. Binary probe 59 contains at its 3' end 66

twenty-three nucleotides complementary to the region 4554-

4576 of HIV RNA, part of target sequence 52. Adjacent to this

10   probe sequence 66 is segment 65, that comprises 158

nucleotides that encode the 3' end of MDV-1 (+) RNA. The

sequence of the two DNA binary probes is shown in Figure 8.

Probe 58 was prepared by the ligation of two

oligodeoxyribonucleotides prepared on a DNA synthesizer.

15   Probe 59 can be similarly prepared. We prepared probe 59,

however, by successively cloning DNA sequences into a

bacteriophage M13 vector. Single-stranded DNA from the phage

was isolated. This DNA was cleaved at the ends of the probe

sequence by restriction endonucleases. The sites of cleavage

20   were made double stranded by hybridization with

oligodeoxyribonucleotides. The cleaved probe sequence was

purified by gel electrophoresis.

These probes are hybridized to the HIV RNA, and the

hybrids are captured, and washed in the same manner as

25   described in Example 1. After washing, the paramagnetic

particles are incubated with RNase H. RNase H degrades those

capture probes that are hybridized to their targets, releasing

the binary probes-target hybrids 70 (shown in Figure 7) into

the supernatant. The hybrids in the supernatant are then

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separated from the paramagnetic particles and transferred to a

new test tube. The hybrids are then incubated with T4 DNA

ligase, in order to link those binary probes that are

correctly hybridized to their targets. After ligation, the

5   hybrids are incubated with the Klenow fragment of E. coli DNA

polymerase and T7 RNA polymerase in order to generate

transcripts 71. These incubations are depicted as separate

steps in Figure 7, for illustration. The combined action of

these enzymes leads to the synthesis of replicatable RNA

10   molecules, which are subsequently amplified exponentially by

incubation with Qβ replicase (as described in Example 1).

Example 3: RNA Target, RNA Binary Probes, DNA or RNA Capture
Probe and Ribozyme Cleavage
15
Our most preferred method of target-dependent release of

reporter probe-target hybrids is cleavage of the targets by

incubation with RNase H. In certain situations, a ribozyme

may be used to cleave the target specifically in the region

20   between the capture probe and the reporter probe or probes.

Although we prefer a "hammerhead" ribozyme (Haseloff &

Gerlach, 1988) for this cleavage, any other ribozyme which can

be targeted towards this region can be used. Figure 9

illustrates one embodiment of this example in which a

25   hammerhead ribozyme is used.

Target RNA 81 contains target sequence 82. A single DNA

capture probe 84, containing hybridization sequence 85 and

biotin moiety 86 is shown attached to paramagnetic particle 87

via its streptavidin coating 88. A pair of RNA binary

30   reporter probes 90, 92 are shown hybridized adjacent to one

- 44 -

another on target sequence 82 via their respective sequences

91, 93. Hybridized to target 81 at a site between capture

probe 84 and reporter probes 90, 92 is hammerhead ribozyme 89,

that contains hybridization sequences 94, 95. Sequences 94,

5   95 are sometimes called "recognition sequences."

The ribozyme 89 is designed such that recognition

sequence 94 (25-30 nucleotides in length) is longer than

recognition sequence 95 (6-7 nucleotides in length). Ribozyme

89 is added as an additional probe. To effect cleavage of the

10   target at 83, the magnesium ion concentration is adjusted to

20 millimolar, and the tubes are incubated for 60 minutes at

55°C. Since recognition sequence 95 is too small to hold the

cleaved target, the reporter probes-target hybrids are

released into the solution. This release is target-dependent.

15   The release is followed by ligation of binary probes 90, 92 by

incubation with T4 DNA ligase. Next, the ligated probes,

i.e., the reporter molecule (not shown) is amplified by

incubation with Qβ replicase.

In an alternative embodiment of this example, an RNA

20   capture probe is used and the head of the capture probe is a

ribozyme (or the head of the capture probe comprises a

ribozyme and an additional sequence that is complementary to

the target).

25   Example 4: RNA Target, DNA Binary Probes, DNA Capture Probe
and RNase III Cleavage with the Use of an RNA Site-Identifying
Probe

The reporter probe-target hybrids can be released in a

30   target-dependent manner with the aid of a 40-50 nucleotide-

long site-identifying probe, which we sometimes call a

"release probe." That probe should be made of RNA and can be
hybridized to the RNA target along with the other probes.
Incubation with RNase III cleaves the target between the
capture probe and the binary reporter probes, releasing the
5   binary probes-target hybrids into solution for amplification.
RNase III cleavage sites have been described in the literature
as specific sequences in double-stranded RNA, but we have
observed that any double-stranded RNA more than 30 nucleotides
long is a good substrate for RNase III. Because of this, a
10   hybrid between an RNA reporter probe and an RNA target is
degraded, and DNA reporter probes and DNA capture probes
should be used.

A preferred design for DNA binary probes is depicted in
Figure 10. Target RNA contains target sequence 102. A single
15   DNA capture probe 104, containing hybridization sequence 105
and biotin moiety 106 is shown attached to paramagnetic
particle 107 via its streptavidin coating 108. A pair of DNA
binary reporter probes 110, 113 are shown hybridized adjacent
to one another on target sequence 102 via their respective
20   sequences 111, 114. Probe 110 includes terminal region 112
that can be extended to form a T7 promoter, similarly to probe
58 of Example 2. Site-identifying RNA probe 59 is shown
hybridized to target 101 between the capture probe and the
reporter probes. Probe 59 is as described above. Incubation
25   with RNase III cleaves target 101 at 103.

We have demonstrated that DNA binary probes such as 110,
113 can be ligated by incubation with T4 DNA ligase in a
target-dependent manner. Amplifiable reporter RNAs are
generated by transcription of the ligated DNA binary probes by

incubation with T7 RNA polymerase. Subsequent amplification

of the reporter RNAs by incubation with Qβ replicase leads to

a detectable signal. When the reporter molecule is a

recombinant MDV-1 RNA, at least, the Klenow fragment of E.

5   coli DNA polymerase is added (and, of course,

deoxyribonucleotides are added) with the T7 RNA polymerase to

complete the promoter, if necessary, and to further extend the

DNA strand to form a double-stranded template.

10   Example 5: RNA Target, DNA Binary Probes, RNA Capture Probe
     and RNase III Cleavage

     Instead of the DNA capture probe described in Example 4,

15   the capture probe can be made of RNA. This makes it

unnecessary to use a site-identifying probe. The capture

probe-target hybrids themselves are substrates for RNase III.

Incubation with RNase III specifically releases the binary

probes-target hybrids. This release is followed by incubation

20   with T4 DNA ligase, T7 RNA polymerase (and possibly the Klenow

fragment of E. coli DNA polymerase, as discussed in Example

4), and Qβ replicase, to generate an amplified signal.

     Example 6: RNA Target, DNA Binary Probes, DNA Capture Probe
25   and RNase A Cleavage

     If the capture probes and the reporter probes are made of

DNA, then it is possible to use any single-strand-specific

30   ribonuclease, such as RNase A, for release. RNase A does not

cleave the target in the probe-target region, because its

normal substrate is single-stranded RNA, but release is still

specific, because target cleavage occurs between the capture

probe and the target sequence. Nonspecifically bound reporter

probes remain on the capture probes, which are permanently

linked to the surface of the particles. Release is followed

by incubation with T4 DNA ligase, T7 RNA polymerase (and

5   possibly the Klenow fragment of E. coli DNA polymerase, as

discussed in Example 4), and Qβ replicase, to generate an

amplified signal.

Example 7: DNA Target, Unary or Binary Probes, DNA Capture
10   Probe and Restriction Endonuclease Cleavage

If the target is DNA, a restriction enzyme can be used.

The sequence of the head of the capture probe is chosen such

15   that it contains a restriction endonuclease recognition site.

Reporter probe-target hybrids are released from the capture

probes by incubating, e.g., the paramagnetic particles with an

appropriate restriction endonuclease. The preferred

embodiment of the reporter probe is a pair of RNA binary

20   probes, as in Example 1, but the reporter probe can be a pair

of DNA binary probes, as in Example 4, or the reporter probe

could be a DNA or RNA unary probe. If binary probes are used,

the released binary probes-target hybrids are then incubated

with T4 DNA ligase and then amplified, directly or indirectly.

25

Example 8: DNA Target, DNA Binary Probes, RNA Capture Probe
and RNase H Cleavage

30   If the target and the reporter probes are DNA, while the

capture probe is made of RNA, RNase H can be used to

specifically release the binary probes-target hybrids from the

capture probes. Release is followed by incubation with T4 DNA

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ligase, T7 RNA polymerase (and possibly the Klenow fragment of

E. coli DNA polymerase, as discussed in Example 4), and Qβ

replicase, to generate an amplified signal.

5   Example 9: Ribozyme Ligase

        This example describes an assay for an RNA target using

RNA binary probes and a "tethered" ribozyme ligase, which is

an RNA molecule comprised of three regions: (1) a ribozyme

ligase sequence; (2) a "holdfast", which is a sequence

10   complementary to a region of the target that is near to the

target sequence; and (3) a "tether", which is a sequence

joining the ribozyme ligase sequence to the holdfast.

Tethered ribozyme ligases are disclosed in Lizardi et al. Ser.

No. 08,005,895 ___ , for Diagnostic Assays for RNA Using RNA

15   Binary Probes, filed on the same date as this application, by

Tyagi, Landegren, Lizardi, Kramer and Szostak. It utilizes

Tetrahymena ribozyme ligase (Doudna and Szostak, 1989) and

special binary probes as disclosed in Lizardi et al. Ser. No.

08,005,895 ___ .

20

        A.   Preparation of Binary Reporter Probes and a Tethered
              Ribozyme Ligase

        The "first probe" is a 71 nucleotide-long RNA generated

25   by in vitro transcription of an artificial gene. The sequence

of this RNA is as follows:

5'-GGGGACCCCCCCGGAAGGGGGGGGACGAGGUGCGGGCACCUCGUACGGGAGUUCGAGCGU

GACGACCGUAGU-3'. The last 9 nucleotides of this RNA form a

"first probe sequence" (underlined) that is complementary to

the sequence 3'-UUGGCAUCG-5', which is part of the HIV-1 RNA

target sequence.

The "second probe" is a 186-nucleotide-long RNA with the

sequence:

5   5'-GACUGGUGAAAUUGCUGCCAUUGUCUGUAGCACGCGCUAGCGCUUUCGCGCUCUCCCAG

GUGACGCCUCGUGAAGAGGCGCGACCUUCGUGCGUUUCGGCAACGCACGAGAACCGCCACGC

UGCUUCGCAGCGUGGCUCCUUCGCGCAGCCCGCUGCGCGAGGUGACCCCCCGAAGGGGGGUU

CCC-3'. The first nucleotide of this RNA is a guanosine,

which does not pair to HIV-1 RNA, but is required for ligation

10   by the <u>Tetrahymena</u> ribozyme ligase (Doudna & Szostak, 1989).

The next 28 nucleotides form a "second probe sequence"

(underlined) that is complementary to the sequence

3'-UGACCACUUUAACGACGGUAACAGACAU-5', which is the other part of

the HIV-1 RNA target sequence adjacent to the 5' end of

15   sequence 3'-UUGGCAUCG-5'.

The second probe is generated by <u>in vitro</u> transcription

of an artificial gene that codes for the desired RNA sequence

and contains a T7 promoter. The artificial gene is generated

by the polymerase chain reaction (Erlich et al., 1991) using

20   the following primers:

5'-TGCGTAATACGACTCACTATAGACTGGTGAAATTGCTGCCATTGTCTGTAGCACGCTGC

TAGCGCTTTCGCGCTCTCC-3' and 5' GGGGAACCCCCCTTCGGGGGGTCACC 3'.

These primers are used in a polymerase chain reaction in the

presence of plasmid pT7MDVHIV20, described at page 1827 of

25   Lomeli et al., 1989, to generate the artificial gene for the

synthesis of the second probe. The 186-nucleotide-long

transcript is purified by polyacrylamide gel electrophoresis.

The tethered ribozyme ligase is generated by <u>in vitro</u>

transcription, as described by Doudna & Szostak (1989). An

artificial gene for the transcription of the tethered ribozyme

ligase is generated by PCR from plasmid pJD1100 (Doudna &

Szostak, 1989) using the following primers:

5'-GCGTAATACGACTCACTATAGGGTTTTTACTGGCCATCTTCCTGCTAATTTTAAGTTGA

5   GAGTTATCAGGCATGCACCTG-3' and 5'-CTAGCTCCCATTAAGGAGAG-3'. The

product of <u>in vitro</u> transcription is 345 nucleotides long and

contains a holdfast having the sequence

5'-GTTTTTACTGGCCATCTTCCTGCTAATTTTAA-3' and a tether having the

sequence 5'-TTTGAG-3', which is connected to the P2 stem of

10   the <u>Tetrahymena</u> ribozyme ligase (Doudna & Szostak, 1989). The

holdfast is complementary to a sequence in HIV-1 RNA that is

five nucleotides removed from the target sequence.

Each binary probe was tested to see if it could be

exponentially replicated by incubation with Qβ replicase, and

15   neither was.

B.   <u>Assay for HIV-1 RNA</u>

The assay is the same as the assay of Example 1, except

that each sample is dissolved in 20 microliters of 5 M GuSCN;

20   then to each sample is added an 80-microliter solution

containing: 10^{13} molecules of each capture probe, 2 x 10^{10}

molecules of first probe, 2 x 10^{10} molecules of second probe,

and 2 x 10^{10} molecules of tethered ribozyme ligase.

Hybridization and washing are carried out in 1 M GuSCN,

25   instead of 2 M GuSCN. Then, instead of washing with 1x ligase

buffer, ribozyme ligase buffer (10 mM NH$_4$Cl, 20 mM MgCl$_2$, 4 mM

spermidine, and 30 mM Tris-HCl, pH 7.4) is used. One unit of

RNase H, dissolved in ribozyme ligase buffer is then added,

and the binary probes-target hybrids are released from the

surface of the paramagnetic particles by a 10-minute
incubation at 37°C. The binary probes-target hybrids are then
isolated from the particles, as described in Example 1. The
isolated binary probes-target hybrids are then incubated for
5   60 minutes at 58°C (Doudna & Szostak, 1989) to ligate the
binary probes in a target-directed fashion. The remainder of
the assay is as described in Example 1.

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5

We claim:

1.   A nucleic acid sandwich hybridization assay to
detect the presence in a sample of a preselected nucleic acid
target containing a target sequence comprising the steps of:

        a.   incubating the sample with a unary reporter
probe under conditions promoting hybridization of the
reporter probe to said target sequence,

        b.   incubating the sample with a capture probe
under conditions promoting hybridization of the capture
probe to said target,

        c.   immobilizing the capture probe on the surface
of a solid,

        d.   washing said solid after steps a, b and c,

        e.   incubating the washed solid from step d with a
liquid containing a cleaving agent capable of cleaving an
immobilized capture probe-target-reporter probe hybrid to
release therefrom a reporter probe-target hybrid,

        f.   separating the liquid from the solid after step
e, and

        g.   detecting the presence of the reporter probe in
the liquid from step f.

2.   An assay according to claim 1 wherein the target is
RNA, the capture probe is DNA, and the cleaving agent is
ribonuclease H.

3.   An assay according to claim 1 wherein the reporter
probe is a template for an RNA-directed RNA polymerase,
additionally comprising the step of exponentially amplifying
said reporter probe after said separating step directly with

an RNA-directed RNA polymerase to form an amplified product,
and wherein said step of detecting comprises detecting the
amplified product.

4.   An assay according to claim 3, wherein said cleaving
agent is ribonuclease H.

5.   An assay according to claim 3, wherein the RNA-
directed RNA polymerase is Qβ replicase.

6.   An assay according to claim 3, wherein said step of
detecting is quantitative.

7.   An assay according to claim 1 additionally
comprising a step of exponentially amplifying after said
separating step.

8.   An assay according to claim 7, wherein said step of
amplifying comprises a reaction selected from the group of
LCR, PCR, 3SR, SDA and TDR.

9.   An assay according to claim 1, wherein the reporter
probe is DNA, additionally comprising the steps of
transcribing the reporter probe to produce an RNA transcript
that is a template for an RNA-directed RNA polymerase and
exponentially amplifying said transcript using the RNA-
directed RNA polymerase, wherein said step of detecting
comprises detecting the presence of amplified transcript.

10.   An assay according to claim 1, wherein said step of
detecting comprises detecting said reporter probe.

11.   An assay according to claim 1, additionally
including a second capture hybridization probe, wherein said
cleaving agent is also capable of cleaving an immobilized
reporter probe-target-second capture probe hybrid to release
therefrom said reporter probe-target hybrid.

12. An assay according to claim 1, wherein the incubations of steps a and b are performed simultaneously, prior to the performance of step c.

13. An assay according to claim 1, wherein the incubations of steps a and b are performed after the performance of step c.

14. An assay according to claim 1, wherein said step of detecting is quantitative.

15. In a nucleic acid sandwich hybridization assay, the improvement comprising immobilizing a reporter probe-target hybrid with a capture probe, washing the immobilized hybrid, cleaving to separate the reporter probe-target hybrid from the capture probe, and isolating the cleaved reporter probe-target hybrid from the capture probe.

16. A nucleic acid sandwich hybridization assay to detect the presence in a sample of a preselected nucleic acid target containing a target sequence comprising the steps of:

a. incubating the sample with a pair of binary reporter probes under conditions promoting hybridization of the reporter probes to said target sequence,

b. incubating the sample with a capture probe under conditions promoting hybridization of the capture probe to said target,

c. immobilizing the capture probe on the surface of a solid,

d. washing said solid after steps a, b and c,

e. incubating the washed solid with a liquid containing a cleaving agent capable of cleaving an

immobilized capture probe-target-reporter probes hybrid

to release therefrom a reporter probes-target hybrid,

   f.   separating the liquid from the solid containing

the capture probe,

   g.   ligating said binary probes in a target-

dependent fashion to form a reporter molecule, and

   h.   generating an amplified, detectable signal.

   17.   An assay according to claim 16, wherein steps e and

f are performed before step g.

   18.   An assay according to claim 16, wherein the

incubations of steps a and b are performed simultaneously,

prior to the performance of step c.

   19.   An assay according to claim 16, wherein the

incubations of steps a and b are performed after the

performance of step c.

   20.   An assay according to claim 16, wherein said binary

hybridization reporter probes are DNA probes and wherein said

step of generating a signal comprises transcribing said

reporter molecule to produce an RNA transcript that is a

template for an RNA-directed RNA polymerase and exponentially

amplifying said transcript using an RNA-directed RNA

polymerase.

   21.   An assay according to claim 20, wherein said

polymerase is $Q\beta$ replicase.

   22.   An assay according to claim 16, where said step

generating an amplified, detectable signal comprises a

reaction selected from the group of LCR, PCR, 3SR, SDA and

TDR.

23. An assay according to claim 16, wherein said target is RNA, wherein said binary hybridization reporter probes are RNA probes, wherein said reporter molecule is a template for a preselected RNA-directed RNA polymerase, and said step of generating a signal includes addition of said RNA polymerase.

24. An assay according to claim 23, additionally comprising the step of quantitatively detecting said signal.

25. An assay according to claim 23, wherein said step of generating a detectable signal includes a molecule that becomes fluorescent when it binds to said reporter molecule.

26. An assay according to claim 23, wherein said RNA polymerase is Qβ replicase.

27. An assay according to claim 23, wherein said step of ligating includes incubating with a ligase selected from the group consisting of DNA ligases and ribozyme ligases.

28. An assay according to claim 23, wherein the capture probe is DNA and the cleaving agent is ribonuclease H.

29. An assay according to claim 28, wherein steps a and b are performed before step d, and wherein steps e and f are performed before step g.

30. An assay according to claim 28, wherein said step of ligating includes incubating with a ligase selected from the group consisting of DNA ligases and ribozyme ligases.

31. An assay according to claim 30, wherein said RNA polymerase is Qβ replicase.

32. An assay according to claim 23, additionally including a second hybridization capture probe, wherein said cleaving agent is also capable of cleaving an immobilized

reporter probes-target-second capture probe hybrid to release therefrom said reporter probes-target hybrid.

33. An assay according to claim 16, additionally including a second hybridization capture probe.

34. An assay according to claim 16 that is quantitative.

35. In a nucleic acid sandwich hybridization assay, the improvement comprising immobilizing a binary reporter probes-target hybrid with a capture probe, washing the immobilized hybrid, cleaving to separate the reporter probes-target hybrid from the capture probe, isolating the cleaved reporter probes-target hybrid from the capture probe, and ligating the reporter probes of the reporter probes-target hybrid in a target-dependent fashion.

36. An assay according to claim 35, wherein the binary probes-target hybrid comprises RNA binary probes.

37. An assay according to claim 35, wherein the binary probes-target hybrid is an RNA binary probes-RNA target hybrid.

38. A kit of reagents for performing a nucleic acid sandwich hybridization assay to detect the presence of a preselected target containing a target sequence, comprising:

a. a mixture including a capture probe for the target and a unary reporter probe for the target sequence, and

b. instructions for performing an assay that includes a step of cleaving an immobilized reporter probe-target-capture probe hybrid with a cleaving agent to release therefrom a reporter probe-target hybrid.

39. A kit according to claim 38 further comprising the cleaving agent.

40. A kit of reagents for performing a nucleic acid sandwich hybridization assay to detect the presence of a preselected target containing a target sequence, comprising:

    a. a pair of binary reporter probes hybridizable to said target sequence,

    b. a capture probe hybridizable to said target, and

    c. instructions for performing an assay that includes a step of washing an immobilized reporter probes-target-capture probe hybrid.

41. A kit according to claim 40, wherein said instructions further prescribe a step of cleaving immobilized reporter probes-target-capture probe hybrid to release therefrom a reporter probes-target hybrid.

42. A kit according to claim 41, wherein said instructions prescribe a step of ligating reporter probes hybridized to said target sequence after said step of cleaving.

43. A kit according to claim 40, wherein said binary reporter probes are RNA probes that, when ligated, produce a reporter molecule that is exponentially amplifiable by an RNA-directed RNA polymerase.

44. A kit according to claim 41 further comprising:

    d. 5 M GuSCN buffer,

    e. ribonuclease H,

    f. T4 DNA ligase, and

    g. Qβ replicase.

45. A kit according to claim 44, wherein the capture probe has a biotinylated tail, further comprising:

    g.   paramagnetic particles coated with streptavidin.

46. A kit according to claim 44, wherein said binary reporter probes are DNA probes, further comprising:

    d.   T7 RNA polymerase,

    e.   the Klenow fragment of E. coli DNA polymerase, and

    f.   Qβ replicase.

47. A kit according to claim 46, further comprising:

    g.   5 M GuSCN buffer, and

    h.   T4 DNA ligase.

48. A nucleic acid hybridization assay to detect the presence in a sample of a preselected nucleic acid target containing a target sequence comprising the steps of:

    a.   incubating the sample with a pair of binary reporter probes under conditions promoting hybridization of the reporter probes to said target sequence,

    b.   incubating the sample with a capture probe under conditions promoting hybridization of the capture probe to the target,

    c.   immobilizing the capture probe on the surface of a solid,

    d.   washing said solid after steps a, b and c,

    e.   ligating said binary probes in a target-dependent fashion to form a reporter molecule, and

    f.   detecting the presence of said reporter molecule.

49. An assay according to claim 48, wherein the incubations of steps a and b are performed simultaneously and prior to the performance of step c.

50. An assay according to claim 48, wherein the incubations of steps a and b are performed after the performance of step c.

51. An assay according to claim 48 additionally comprising a step of exponentially amplifying to produce an amplified product, wherein said step of detecting comprises detecting the amplified product.

52. An assay according to claim 51, wherein said step of amplifying comprises a reaction selected from the group of LCR, PCR, 3SR, SDA and TDR.

53. An assay according to claim 51, wherein said step of detecting is quantitative.

54. An assay according to claim 51, wherein the reporter molecule is DNA and wherein said step of amplifying includes transcribing the reporter molecule to form an RNA transcript and amplifying the RNA transcript with an RNA-directed RNA polymerase.

55. An assay according to claim 54, wherein the RNA-directed RNA polymerase is Qβ replicase.

56. An assay according to claim 51, wherein the step of amplifying includes amplification by a ligase chain reaction.

57. An assay according to claim 51, wherein the step of amplifying includes amplification by a polymerase chain reaction.

58. An assay according to claim 51, wherein the reporter molecule is a template for an RNA-directed RNA polymerase and

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wherein said step of amplifying comprises amplifying the
reporter molecule directly with an RNA-directed RNA
polymerase.

    59. An assay according to claim 58, wherein said target
5   is RNA.

    60. An assay according to claim 59, wherein the RNA-
directed RNA polymerase is Qβ replicase.

    61. An assay according to claim 59, wherein said step of
ligating comprises incubating with a ligase selected from the
10   group consisting of DNA ligases and ribozyme ligases.

    62. An assay according to claim 51 additionally
comprising the step of performing reversible target capture
after step d and prior to step e.

    63. In a nucleic acid sandwich hybridization assay, the
15   improvement comprising immobilizing a binary reporter probes-
target hybrid with a capture probe and washing the immobilized
hybrid.

    64. An assay according to claim 63, wherein the binary
probes-target hybrid is an RNA binary probes-RNA target
20   hybrid.

    65. A nucleic acid sandwich hybridization assay for
detecting a preselected nucleic acid target in a sample,
comprising the steps of:

        a.   immobilizing the target on the surface of a
25   solid by means of a capture probe,

        b.   incubating with a cleaving agent to cleave
        capture probe-target hybrids,

        c.   isolating the solid to separate the target from
        the solid and from the capture probe,

d. generating an exponentially amplified signal from the separated target by a process selected from the group of LCR, PCR, 3SR, SDA and TDR, and

e. detecting the product.

66. An assay according to claim 65, wherein the cleaving agent is ribonuclease H.

67. An assay according to claim 65, wherein the target is RNA, the capture probe is DNA, and the cleaving agent is ribonuclease H.

68. An assay according to claim 65 that includes RNA binary probes and an RNA-directed RNA ligase selected from the group consisting of protein ligases and ribozyme ligases.

69. An assay according to claim 68, wherein the target is RNA, the capture probe is DNA, and the cleaving agent is ribonuclease H.

70. An assay according to claim 69, wherein the ligase is T4 DNA ligase.

71. A kit of reagents for performing a nucleic acid sandwich assay for a preselected target, comprising:

a. a capture probe hybridizable with the target, and

b. instructions for performing an assay that includes a step of cleaving an immobilized capture probe-target hybrid to release therefrom the target and generating an exponentially amplified signal.

72. A kit according to claim 71 further comprising:

c. ribonuclease H.

73. A kit according to claim 71, wherein the target is
RNA, further comprising RNA LCR probes and an RNA-directed RNA
ligase.

74. A kit according to claim 73, wherein said ligase is
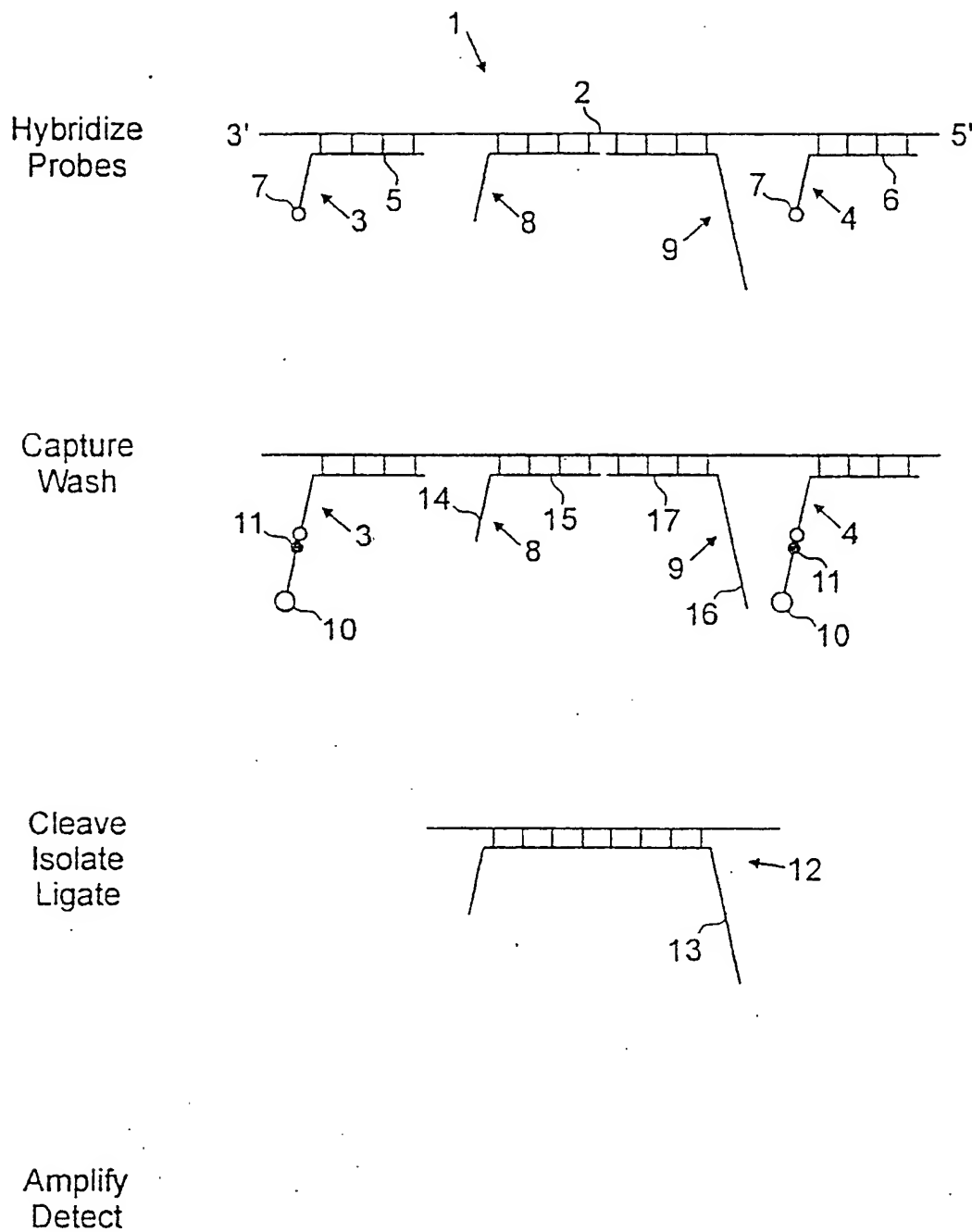5   T4 DNA ligase.

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FIG. 1

Capture Probe 3

```
          10         20         30         40         49
5' Biotin-TACGACTGCT ACCAAGATAA CTTTTCCTTC TAAATGTGTA CAATCTAGC 3'
          _____   _____ _____ _____ _____
```

Capture Probe 4

```
          10         20         30         40         49
5' Biotin-TACGATGTCT GTTGCTATTA TGTCTACTAT TCTTTCCCCT GCACTGTAC 3'
          _____     _____ _____ _____ _____
```

Binary Probe 8

```
      10         20         30         40         50         60
5' GGGGACCCCC CCGGAAGGGG GGGACGAGGU GCGGGCACCU CGUACGGGAG UUCGACCGUG

      70         80         90
   ACGCUCUAGC AGGCGGCCUU AACUGUAGUA CU 3'
   _  _____ _____ __
```

Binary Probe 9

```
      10         20         30         40         50         60
5' GGUGAAAUUG CUGCCAUUGA GAUCUAGAGC ACGGGCUAGC GCUUUCGCGC UCUCCCAGGU
   _____ _____

      70         80         90        100        110        120
   GACGCCUCGU GAAGAGGCGC GACCUUCGUG CGUUUCGGUG ACGCACGAGA ACCGCCACGC

     130        140        150        160        170        180
   UGCUUCGCAG CGUGGCUCCU UCGCGCAGCC CGCUGCGCGA GGUGACCCCC CGAAGGGGGG

      185
   UUCCC 3'
```
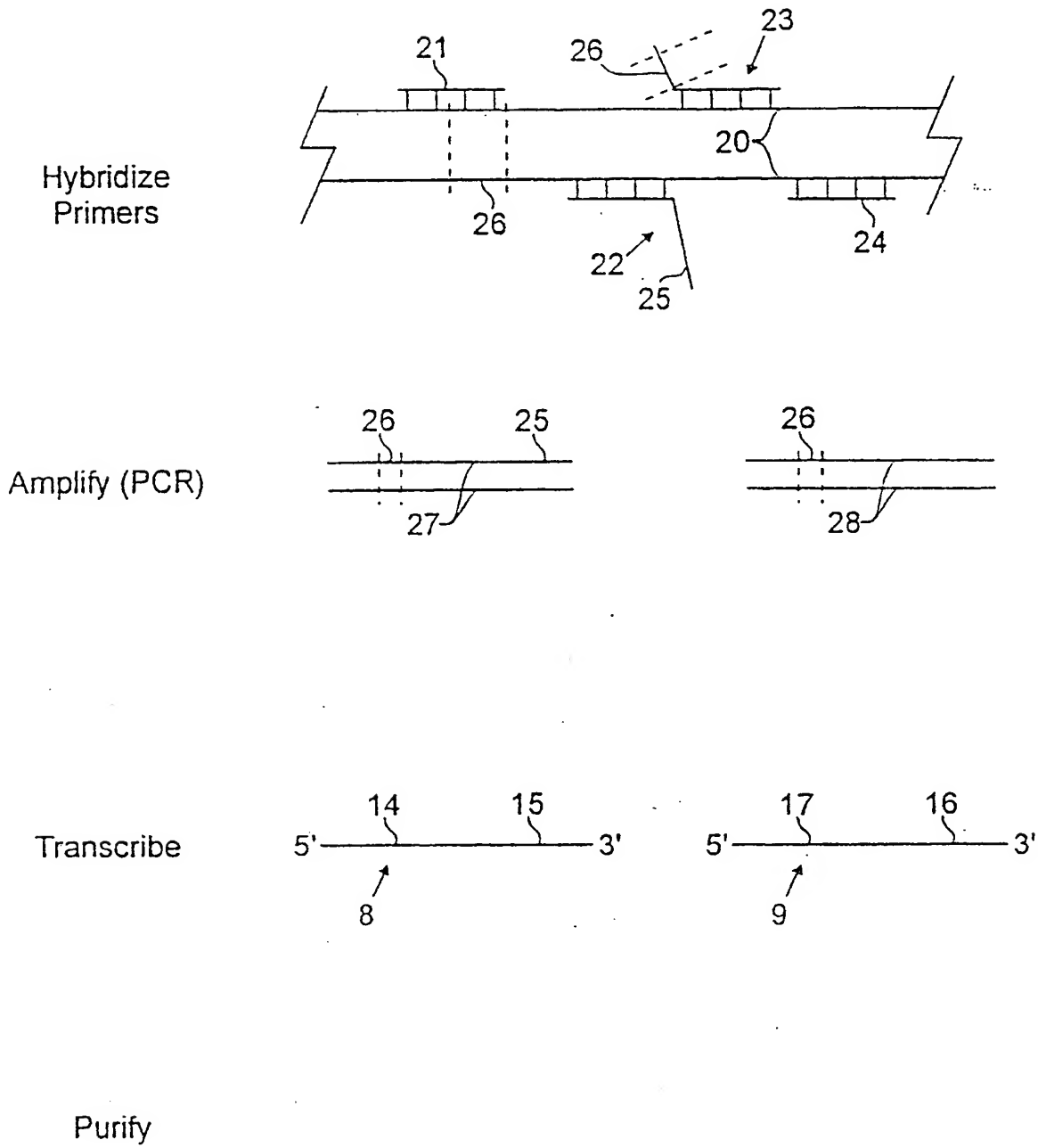
FIG. 2

Hybridize Primers

Amplify (PCR)

Transcribe

5'————3'   5'————3'

Purify

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

Capture Probe 53

```
                10         20         30         40         49
5' Biotin-GGCGACUGCU ACCAAGAUAA CUUUUCCUUC UAAAUGUGUA CAAUCUAGC 3'
          --------  ----------  ----------  ----------  ---------
```

Capture Probe 54

```
                10         20         30         40.        49
5' Biotin-GGCGAUGUCU GUUGCUAUUA UGUCUACUAU UCUUUCCCCU GCACUGUAC 3'
          --------  ----------  ----------  ----------  ---------
```

Binary Probe 58

```
    60                    67
  G-G                ┌──────────────────────────┐
 G   G  CGTTATCTCG AC│TAATAC 3'                 │
 G      GCAATAGAGC TG│ATTATGCT  GAGTGATATC│ CCCTGGGGGG GCCTTCCCCC CCTGCTCCAC
  T-G                └──────────────────────────┘

        GCCCGTGGAG CATGCCCTCA AGCTGGCACT GAATTTTAAT CGTCCTTCTA CC 5'
                                         ---------- ---------- --
```
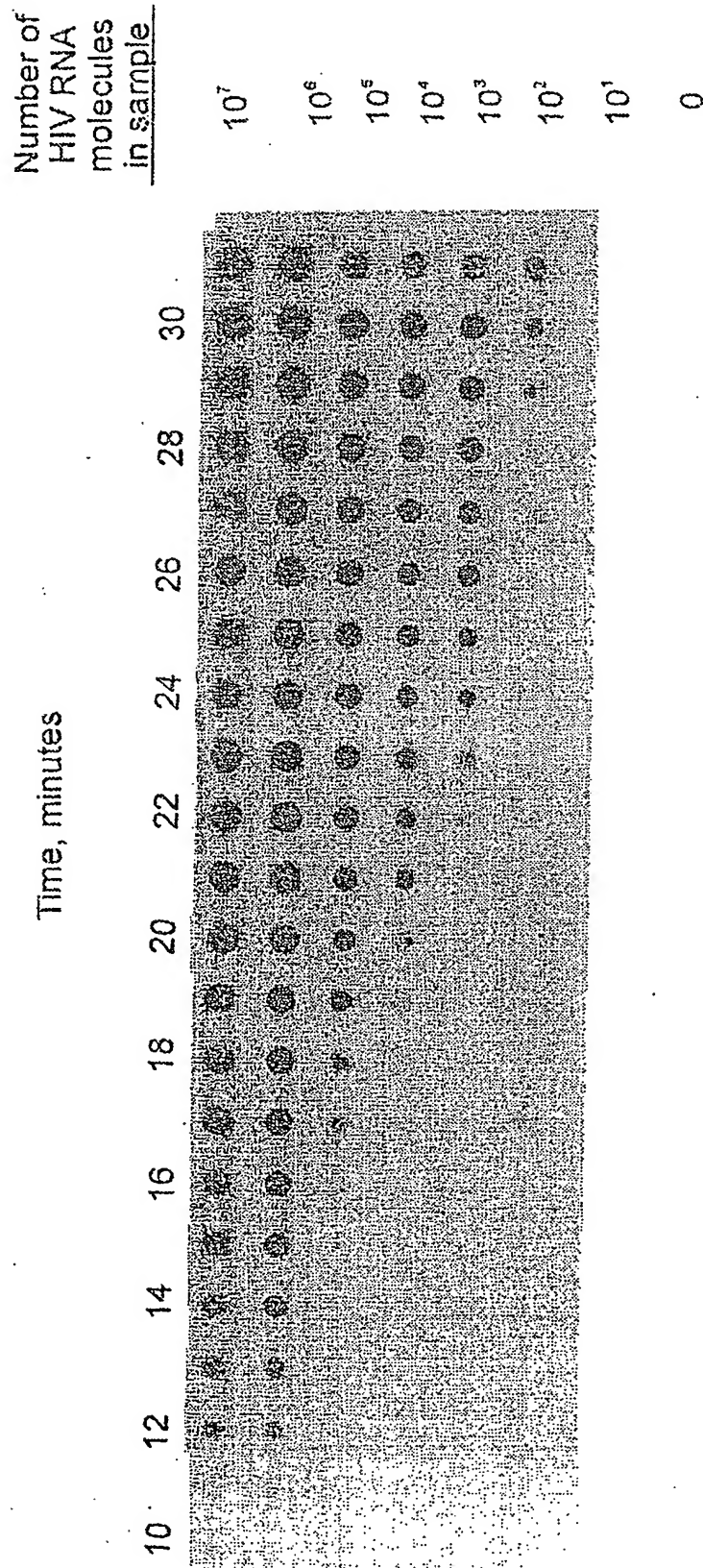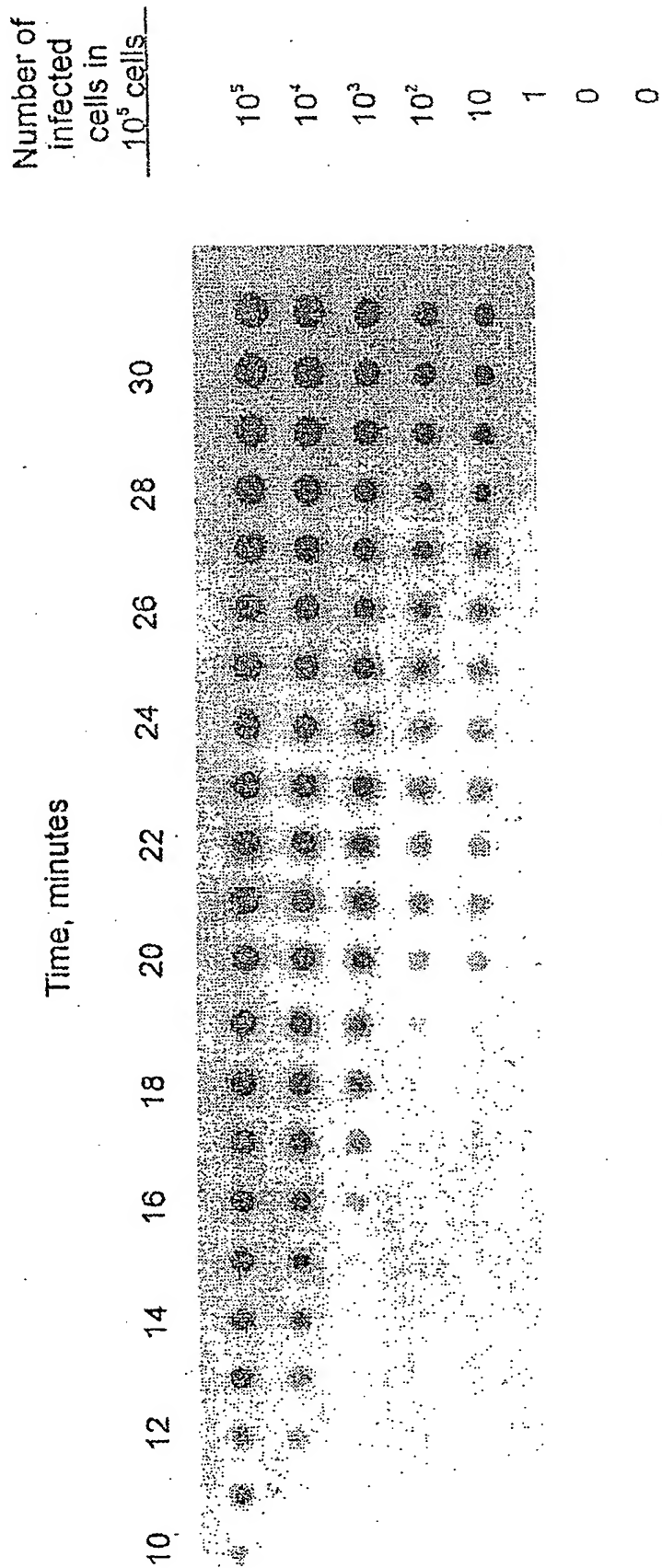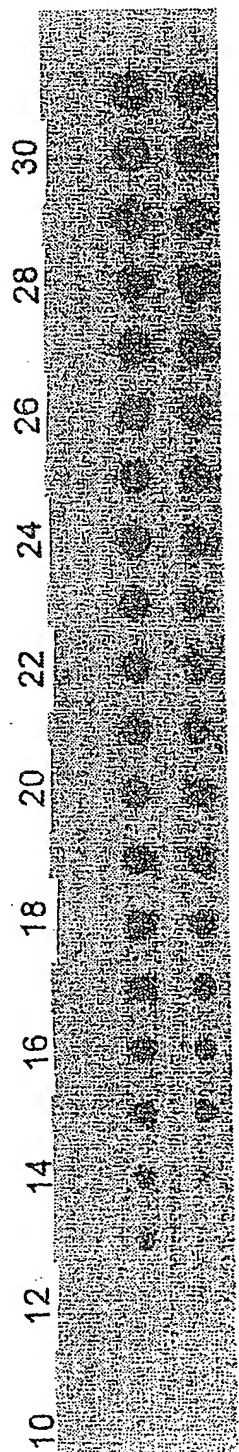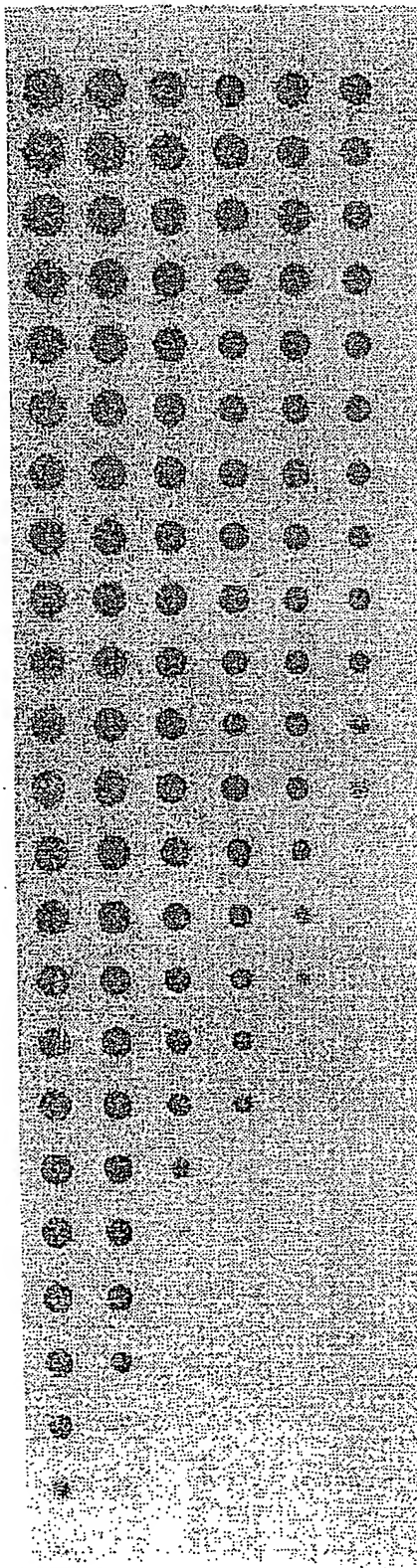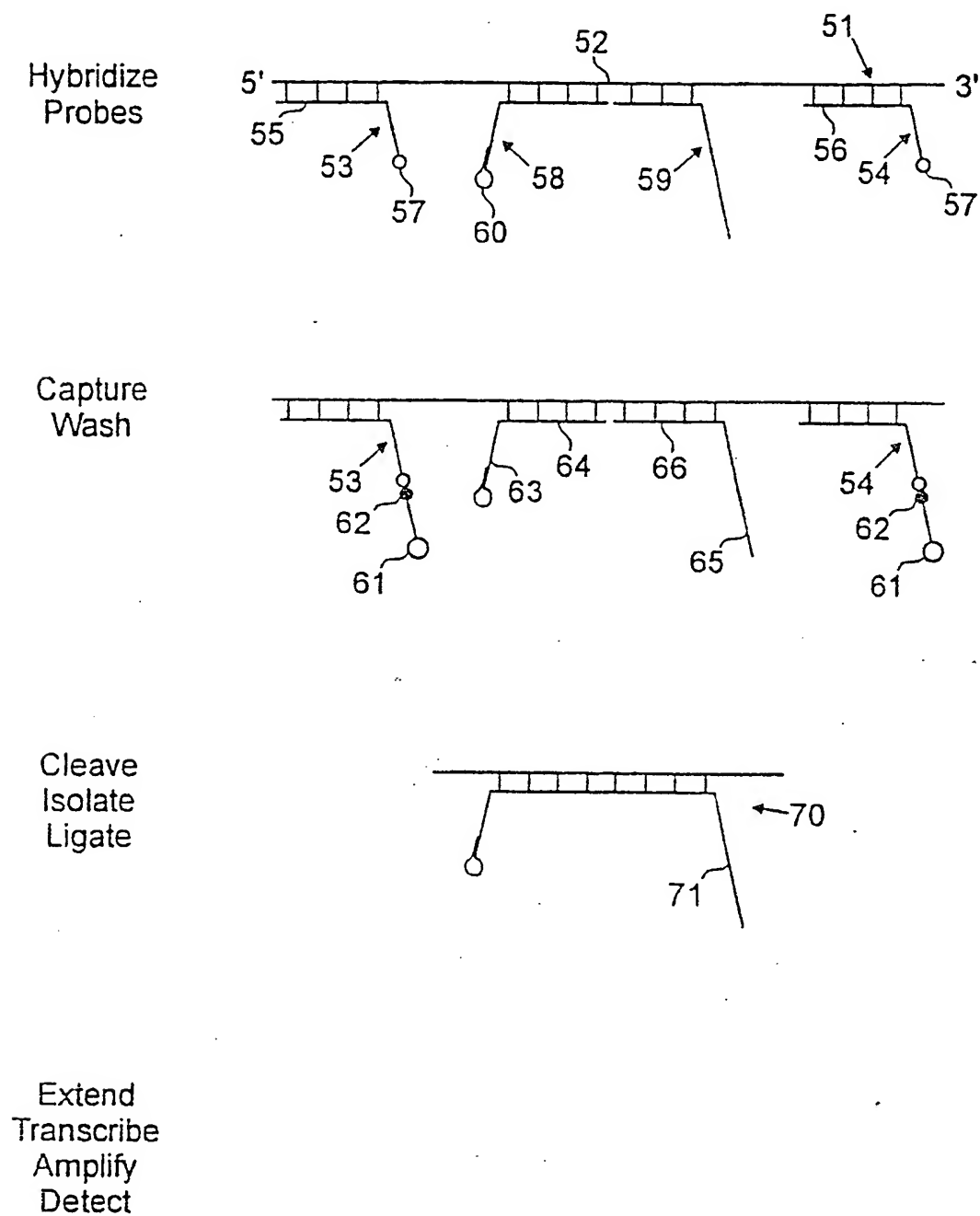
Binary Probe 59

```
3' GGTCATTTTT GTCAAGTATG TCTCAGTGCC CGATCGCGAA AGCGCGAGAG GGTCCACTGC
   ---------- ---------- ---

   GGAGCACTTC TCCGCGCTGG AAGCACGCAA AGCCACTGCG TGCTCTTGGC GGTGCGACGA

   AGCGTCGCAC CGAGGAAGCG CGTCGGGCGA CGCGCTCCAC TGGGGGGCTT CCCCCCAAGG

   G 5'
```
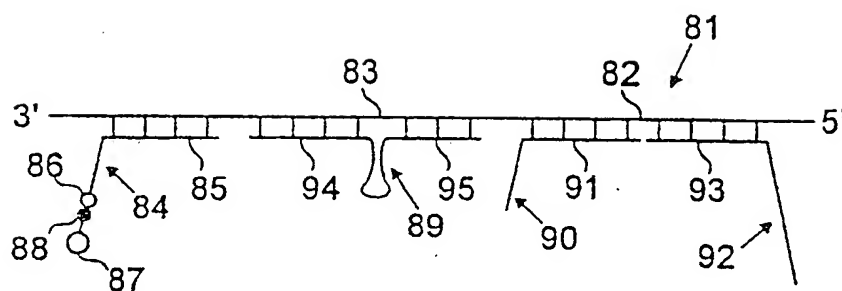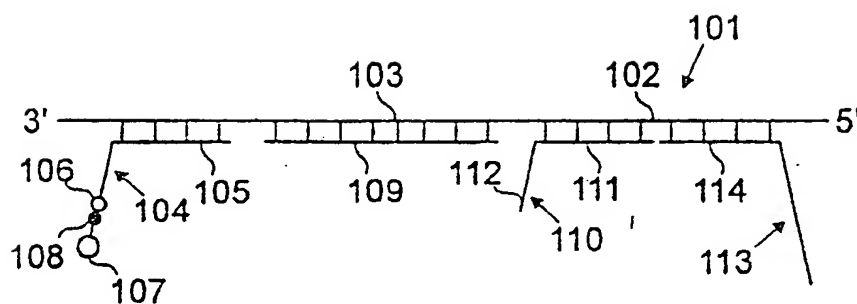
FIG. 8

FIG. 9

FIG. 10

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12P 19/34; C07H 21/04

US CL :435/6, 91.2; 536/24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science, USA; Volume 88, issued January 1991, Barany, "Genetic disease detection and DNA amplification using cloned thermostable ligase", pages 189-193, see especially page 190, Figure 1 and caption.	51 and 56
Y	Cell, Volume 57, issued 05 May 1989, Konarska et al, "Replication of RNA by the DNA-Dependent RNA Polymerase of Phage T7", pages 423-431, see entire document.	46-47

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 FEBRUARY 1994 .	APR 04 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MINDY B. FLEISHER Jill Warden for
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 241, issued 26 August 1988, Landegren et al, "A Ligase-Mediated Gene Detection Technique", pages 1077-1080, see especially page 1077, abstract and column 3, first full paragraph).	16-37 and 40-64
Y	Analytical Biochemistry, Volume 169, issued 1988, Matthews et al, "Analytical Strategies for the Use of DNA Probes", pages 1-25, see especially page 17, section 4.2.1.	12, 18, 25, 45, 48-64
Y	US, A, 4,683,202 (Mullis) 28 July 1987, see especially column 1, lines 13-16 and claims 1-2.	7-8, 22, 51-55, 57-62 and 65-74
X Y	US, A, 4,775,619 (Urdea) 04 October 1988, see especially abstract and Figure 1, embodiment 2B.	1, 10-11, 13-15 2-9, 12; 16-47, 65-74
Y	Nucleic Acids Research, Volume 14, Number 14, issued 1986, Chu et al, "Synthesis of an amplifiable reporter RNA for bioassays", pages 5591-5603, see especially page 5591, abstract.	3-6, 9, 20-21, 23-32, 43-47, 54-55, 58-61
A	Nature, Volume 33, Number 6223, issued 01 June 1989, KRAMER, et al., "Replicatable RNA reporters", pages 401-402, see entire document.	

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT